**Universidade de Aveiro** Departamento de Biologia
**Ano 2013**

**Tânia Raquel Domingues Almeida**

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Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Maria da Conceição Lopes Vieira dos Santos, Professora Associada com agregação do Departamento de Biologia da Universidade de Aveiro e sob co-orientação científica da Doutora Sónia Cláudia Morgado Gonçalves, Investigadora Principal do Centro de Biotecnologia Agrícola e Agro-Alimentar do Alentejo.

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**palavras-chave**

Sobreiro, cortiça, felogénio, suberina, fator de transcrição, R2R3-MYB, splicing alternativo, stresse abiótico, seca, temperatura elevada, recuperação, fito-hormonas, expressão génica, PCR em tempo-real.

**Resumo**

A cortiça é um material natural e renovável obtido de forma sustentável a partir do sobreiro (*Quercus suber* L.) ao longo do ciclo de vida da árvore. A formação da cortiça deriva do crescimento secundário resultante da atividade do câmbio cortical. No entanto, apesar da sua importância económica, o conhecimento dos mecanismos moleculares subjacentes à regulação do desenvolvimento e diferenciação da cortiça é ainda limitado. O trabalho desta Tese de Doutoramento teve como objetivo a caracterização de um fator de transcrição da família R2R3-MYB, o QsMYB1, anteriormente identificado como estando potencialmente envolvido na rede regulatória do desenvolvimento da cortiça.

O primeiro capítulo faz uma introdução ao sobreiro e ao crescimento secundário, com especial ênfase na biossíntese da cortiça. São ainda descritos alguns estudos referentes à regulação do crescimento secundário ao nível da transcrição. São também introduzidas a superfamília de fatores de transcrição MYB e a família R2R3-MYB (em particular). No Capítulo II é apresentada a estrutura completa do gene *QsMYB1*, com identificação de duas variantes de "splicing" alternativo. São ainda descritos os resultados da análise de expressão do gene *QsMYB1*, realizada por PCR em tempo real, em vários tecidos e orgãos de sobreiro. O Capítulo III é dedicado ao estudo da influência de stresses abióticos (seca e temperatura elevada) e recuperação, nos níveis de expressão do gene *QsMYB1*. Os efeitos da aplicação exógena de fito-hormonas, no perfil de expressão de *QsMYB1,* são apresentados no Capítulo IV. No Capítulo V é descrita a abordagem de genética reversa com vista à sobreexpressão do gene *QsMYB1* em linhas transgénicas de *Populus tremula* L. x *tremulóides* Michx. Finalmente, no Capítulo VI são apresentadas as conclusões finais desta Tese e são apontadas algumas linhas de investigação futura baseadas nos resultados obtidos.

**Keywords**

Cork oak, cork, phellogen, suberin, transcription factor, R2R3-MYB, alternative splicing, abiotic stress, drough, high temperature, recovery, phytohormones, gene expression, real-time PCR.

**Abstract**

Cork is a natural and renewable material obtained as a sustainable product from cork oak (*Quercus suber* L.) during the tree's life. Cork formation is a secondary growth derived process resulting from the activity of cork cambium. However, despite its economic importance, only very limited knowledge is available about the molecular mechanisms underlying the regulation of cork biosynthesis and differentiation. The work of this PhD thesis was focused on the characterization of an R2R3-MYB transcription factor, the QsMYB1, previously identified as being putatively involved in the regulatory network of cork development.

The first chapter introduces cork oak and secondary growth, with special emphasis on cork biosynthesis. Some findings concerning transcriptional regulation of secondary growth are also described. The MYB superfamily and the R2R3-MYB family (in particular) of transcription factors are introduced. Chapter II presents the complete *QsMYB1* gene structure with the identification of two alternative splicing variants. Moreover, the results of *QsMYB1* expression analysis, done by real-time PCR, in several organs and tissues of cork oak are also reported. Chapter III is dedicate to study the influence of abiotic stresses (drought and high temperature) and recovery on *QsMYB1* expression levels. The effects of exogenous application of phytohormones on the expression profile of *QsMYB1* gene are evaluated on Chapter IV. Chapter V describes the reverse genetic approach to obtain transgenic lines of *Populus tremula* L. *x tremulóides* Michx. overexpressing the *QsMYB1* gene. Finally, in Chapter VI the final conclusions of this PhD thesis are presented and some future research directions are pointed based on the obtained results.

## INDEX

# CHAPTER I

## A general introduction

## 1. Cork oak - the National tree of Portugal

Cork oak (*Quercus suber* L.) is considered as one of Portugal's greatest natural treasures. This slow growing ever-green oak belongs to *Fagaceae* family and is widely distributed in the coastal regions of western Mediterranean basin (Portugal, Spain, Southern France, part of Italy and North Africa). Cork oaks are polymorphic and differ in traits such as tree form, flowering periods, flowers and fruits, and form and size of leaves (Pereira 2007). This diversity is often attributed to the interspecific hybridisation that occurs, namely with *Quercus ilex* (holm oak) (e.g. Burgarella et al. 2009). Fig. 1 shows examples of flowers, leaves and fruits of cork oak.

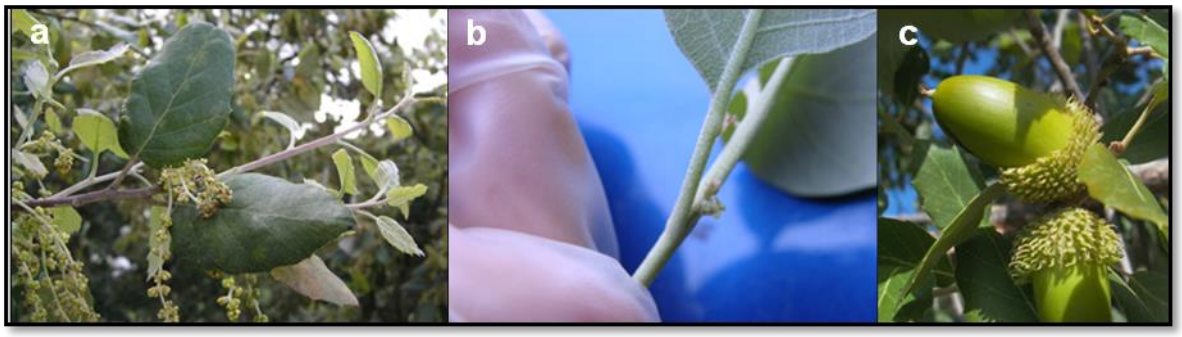


**Fig. 1.** Examples of cork oak organs: **(a)** Cork oak male flowers and leaves; **(b)** female flowers; **(c)** and acorns Accessed in November 2013 in:

(a) http://www.florestar.net/sobreiro/sobreiro.html
(b) http://www.fct.pt/emfoco/sobreiro/foto.phtml.pt?photoref=DSC00054
(c) http://atnatureza.blogspot.pt/2011/01/apoiem-campanha-sobreiro-simbolo.html

Cork oak tree requires a great deal of sunlight and a highly unusual combination of low rainfall and somewhat high humidity. However it shows a high plasticity and is able to adapt its phenology and physiological activity to changing environmental conditions such as severe summer drought and high temperatures (Silva et al. 2005, Pereira 2007).

This species is commonly grown in agro-forestry systems and the most extensive stands are on the Atlantic coast of the Iberian Peninsula, where they are known as *montado* in Portugal and *dehesa* in Spain. These are open woodlands with low tree density (50–300 trees/ha) (Gil and Varela 2008) (Fig. 2). The importance of *Q. suber* species lies primarily with its natural ability of producing cork as a thick outer layer around stems and branches (Leal et al. 2008). In association with cork production, cork oak forests are many times used for cattle grazing, hunting and other non-wood productions (Pereira 2007). Apart of its economic value, cork oak ecosystems comprise also biodiversity, environmental and ecological values, making them one of the few examples of sustainable forestry exploitations (APCOR 2009).


**Fig. 2.** Typical cork oak agro-forestry system (*montado*).

Despite cork oak importance, the species is nowadays threatened by improper policies of land use, biotic stresses, climate change and fire and consequently, an overall decline of cork oak is observed. This situation urges the need for advanced knowledge on the species.

## 2. Secondary growth

Due to their sessile status, the development of higher plants is especially dependent of both physiological and external stimuli. Consequently, plants had to evolve dynamic mechanisms able to cope with these demanding conditions. The capacity to establish pluripotent and proliferating tissues – the meristems - from differentiated cells represents a remarkable example of this developmental plasticity (Agusti et al. 2011). Lateral meristems (vascular and cork cambia) are responsible for plant (gymnosperm and dicotyledonous angiosperms) secondary growth that leads to the girth increase of stem, branches and roots (Raemdonck et al. 2005). This growth in thickness provides mechanical support and has important functions in transport, storage and defence against pathogens (Yordanov et al. 2011). The vascular cambium derives from procambium and contains initials cells that undergo periclinical divisions producing two types of daughter cells, phloem mother cells to the outside of the stem and xylem mother cells to the inside. These mother cells may continue to divide periclinically and proceed through differentiation in secondary xylem (wood) or secondary phloem (part of the bark) (Du et al. 2010). See the structure of a woody stem in Fig. 3. The wood formation comprises a series of sequential biological stages after differentiation that includes extensive cell enlarging, massive secondary wall thickening, cell aging and death (Li et al. 2006).

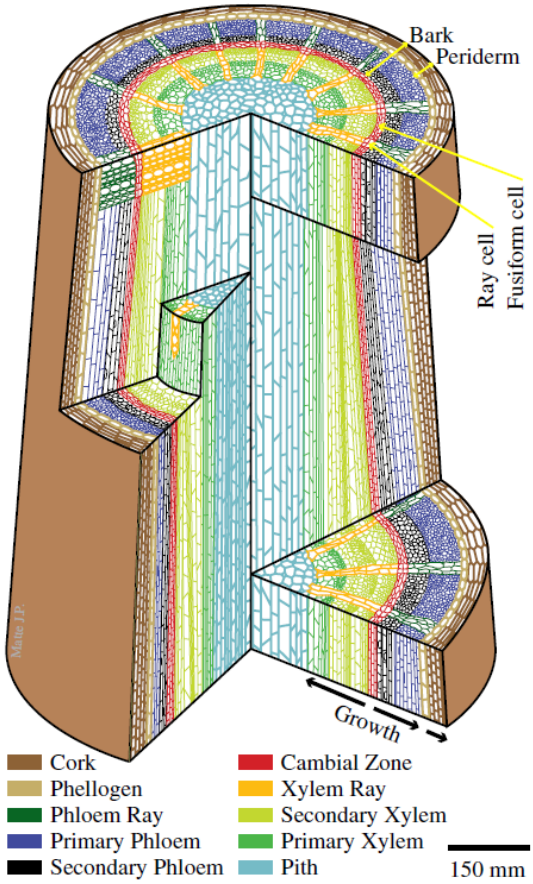


**Fig. 3.** Internal structure of a woody plant stem (taken from Risopatron et al. 2010).

The other lateral meristem, the phellogen or cork cambium, in woody roots initiates usually in the pericycle, while in stem (depending on the species) it can initiate in the epidermis, subepidermis or in much deeper layers that become meristematic (Pereira 2007, Lev-Yadun 2011). In cork oak, the phellogen initiates in the cell layer immediately below the epidermis during the first year of stem growth (Graça and Pereira 2004). Phellogen initials divide periclinically producing phellem (or cork) to the exterior and phelloderm to the interior; these tissues comprise the periderm, a major component of the bark (Beck 2010). The phelloderm cells are living cells with non-suberized walls resembling cortical parenchyma. The phellem cells are dead at maturity and are characterized by a relatively thick layer containing suberin deposited internally to the primary cell wall (Pereira 2007).

In species of *Betula, Fagus, Abies, Carpinus* or *Quercus* the first periderm may be retained for life or for many years (Evert 2006). But, in most trees one periderm is only functional during a limited period and it is replaced by a new functional periderm located to the inner side causing the isolation of its outward tissues from nutrients and water and their death as consequence (Pereira 2007, Lev-Yadun 2011). This complex tissue region of periderms and enclosed non-living tissues is called the rythidome or outer bark (Beck 2010). Environmental conditions influence the appearance of both the initial and sequent periderms. Availability of water, temperature and intensity of light are

factors affecting the timing of periderm development (Evert 2006). Wound sites are also underlain by a periderm, which develops following initiation of a phellogen through dedifferentiation in parenchyma cells bellow the wound (Beck 2010).

## 3. Cork - a natural, renewable and valuable product

Cork is a biological material with remarkable and unique properties (i.e. low density, reduced permeability to liquids and gases, chemical and biological inertia, mechanical elasticity, and insulation properties) (Pereira 2013), making it a valuable product with multiple applications, among which, the bottle stoppers manufacturing is the most well known. Portugal is the world leader in cork production (APCOR 2009).

The debarking occurs when phellogen is highly active (summer) and the young cells are turgid and fragile, allowing the easy separation of cork layers (Silva et al. 2005, Pereira 2007) (Fig. 4).


**Fig. 4.** Cork extraction.

Cork produced in the first periderm, the virgin cork, is commonly removed when tree is about 20-30 years-old. Virgin cork is irregular in structure, thickness and not suited for cork stoppers production (Silva et al. 2005). Following the removal of the first periderm, there is a rapid differentiation of a new phellogen in the outer phloem that will originate a traumatic periderm as a wound response to the death of the initial phellogen and the unprotected exposure to the environment of the living tissues from the phloem (Graça and Pereira 2004, Pereira 2007). After the first cork extraction, the debarking occurs periodically at intervals of 9-10 years. The cork

produced by the second periderm, the second cork, although more regular than virgin, still has an insufficient quality for stoppers production. The cork from the 3rd extraction and following is termed reproduction cork and is the one commonly used by stoppers' industry. See in Fig. 5 examples of high and low quality cork.

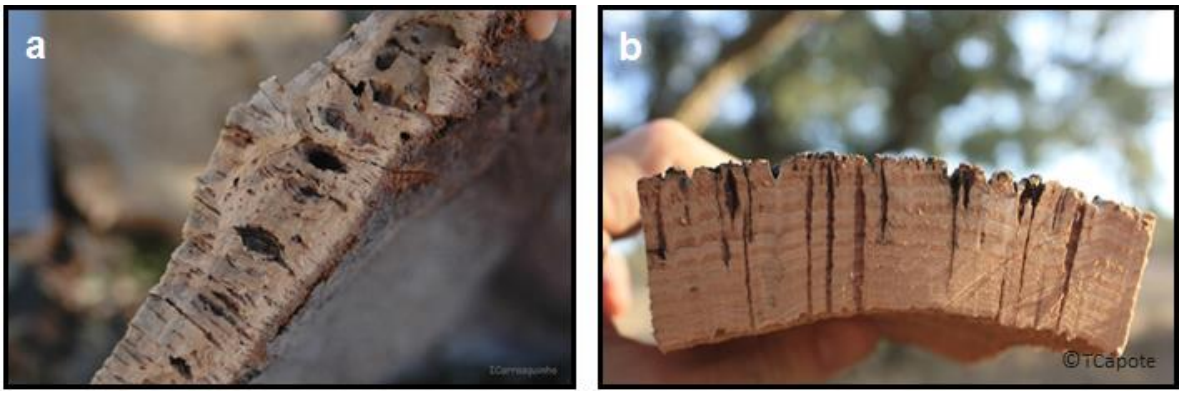


**Fig. 5.** Examples of cork of **(a)** low and **(b)** high quality.

### 3.1. Cork chemical composition and cellular structure

Cork properties are the result of its cellular structure and chemical composition of cell wall (Pereira 2013). Cork chemical composition has been studied by several authors whose results show some variation (Pereira 1988, Bento et al. 2001, Lopes et al. 2001, Pereira 2013). In the most recent work, Pereira (2013) analyzed cork samples from six regions of Portugal and obtained an overall mean chemical composition encompassing suberin as the main structural component (approximately 43%) and lignin (approximately 22.0%) as the second most important constituent. Hence, the remarkable cork properties should be mainly due the combined presence of these two polymers. Both suberin and lignin are known to be essential for plant normal growth and development and are also deposited in response to environmental stimuli (e.g. drought, salt stresses or oxygen deficiency) (Franke and Schreiber, Moura et al. 2010). Other components of cork are extractives (e.g. terpenoids and phenolics) and polysaccharides (e.g. cellulose and hemicellulose).

The cellular structure of cork shows a primary wall, a secondary wall and sometimes a tertiary wall (Fig. 6). Polysaccharides and some polyaromatics are present in the primary and tertiary walls; suberin is located in the secondary wall. Some studies have also reported the presence of extractives associated with suberin in the secondary cell wall (Graça and Santos 2007). Recently, Teixeira and Pereira (2010) observed cork cells from periderms of *Q. suber*, *Quercus cerris*, *Solanum tuberosum* and *Caloptropis procera* by transmission electron microscopy (TEM). The authors did not observe defined lamellae in the secondary wall of various *Q. suber* samples and the same result was obtained for cork cells from *Q. cerris*. By contrast, in potato and *Calotropis* bark they found a lamellar structure with dark and light bands as previously described by other

authors for potato (Serra et al. 2009). Therefore, Teixeira and Pereira (2010) hypothesized that the chemical composition of suberin, which differs between plant species, may be influencing the cell wall topochemistry.

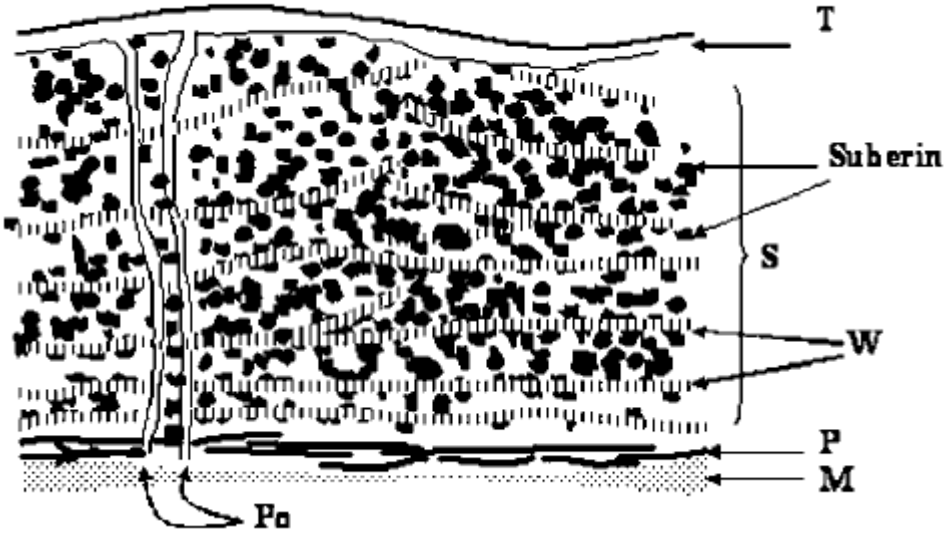


**Fig. 6**. Structure of cork cell wall: (T) tertiary wall, (S) secondary wall, (W) suberin and waxes, (P) primary wall, (M) medium lamella, (Po) pore (taken from Silva et al. 2005).

The use of the term “suberin” is far from a consensus. Classically, suberin was defined as an aliphatic substance that was found to be a polyester and this is still an usual assumption when referring to suberin. However, from the breakdown of the macromolecular suberin structure, there is also a simultaneous release of aromatic residues, leading to the consideration that suberin, besides the aliphatic domain, also has an aromatic domain, which are apparently cross-linked (Pereira 2007). Thus, some authors use the denomination of “aromatic suberin” (for the polyaromatic lignin-like domain) and “aliphatic suberin” (for the fatty acid-derived domain) (Bernards and Razen 2001, Bernards 2002). Other authors make the option of using the term suberin considering only the aliphatic structural component of cork cell wall (Graça and Pereira 2000, Pereira 2007, Pereira 2013). Fig. 7 shows a general biosynthetic scheme for the precursors of suberin polyaliphatic and polyaromatic domains. The quantification of suberin refers to the aliphatic compounds released by depolimerisation, which includes also the released associated aromatic moieties (Graça and Pereira 2000, Pereira 2007). The aliphatic suberin is primarily composed by the monomers: ω-hydroxyacids, long-chain α,ω-dicarboxylic acids and glycerol. Small quantities of alcohols and unsubstituted fatty acids are also present (Franke and Schreiber 2007). Several works have shown that the proportion of each component differs among suberin model species, such as in cork oak bark (Bento et al. 2001, Graça and Santos 2006) potato tuber periderm (Graça and Pereira 2000, Bernards and Razen 2001) or *Arabidopsis* roots (Franke 2005).

Lignin is the second most abundant plant biopolymer after cellulose and is essential for structural integrity of cell wall, providing stiffness and mechanical strengthen to vascular plants. Research on the chemical structure of lignin in suberized cells is scarce (Pereira 2007), thus most of what is known relates to wood lignin. Lignin is a phenylpropanoid heteropolymer derived mainly from three hydroxycinnamyl alcohol monomers (or monolignols) differing in their degree of methoxylation: p-coumaryl, coniferyl, and sinapyl alcohols (Boerjan et al. 2003). These units when incorporated into lignin polymer are called p-hydroxyphenyl (H), guaiacyl (G) and syringyl (S) monolignols. Like in suberin, the proportion of the lignin constituents can vary among plant taxa and species and also among cell type (Vanholme et al. 2010). Cork lignin is a G-type lignin containing 95% G units (Pereira 2007). Monolignols are synthesized through the phenylpropanoid pathway which is shared by other secondary metabolites, such as flavonoids, coumarins (Zhao and Dixon 2011) and also suberin. While monolignol biosynthesis is quite well understood, the lignin assembling, namely the transport of lignin precursors, their deposition and subsequent activation and polymerization is not fully elucidated (Liu 2012). From a structural perspective, the aromatic domain of suberin is unique and distinct from wood lignin. Suberized tissues appear to have a lower proportion of monolignol and a higher content of hydroxycinnamic acids than expected for a lignified tissue (Bernards and Lewis 1998, Bernards 2002). Indeed, there are evidences that two types of polyaromatics may be present in suberized cell walls: one, associated with the polysacharides in the primary (and tertiary) that will be a “true” lignin; the other associated with the aliphatic suberin in the secondary cell wall, will be a polymer either based on the ferulic acid or a yet to be elucidate structure (Graça and Santos 2007).

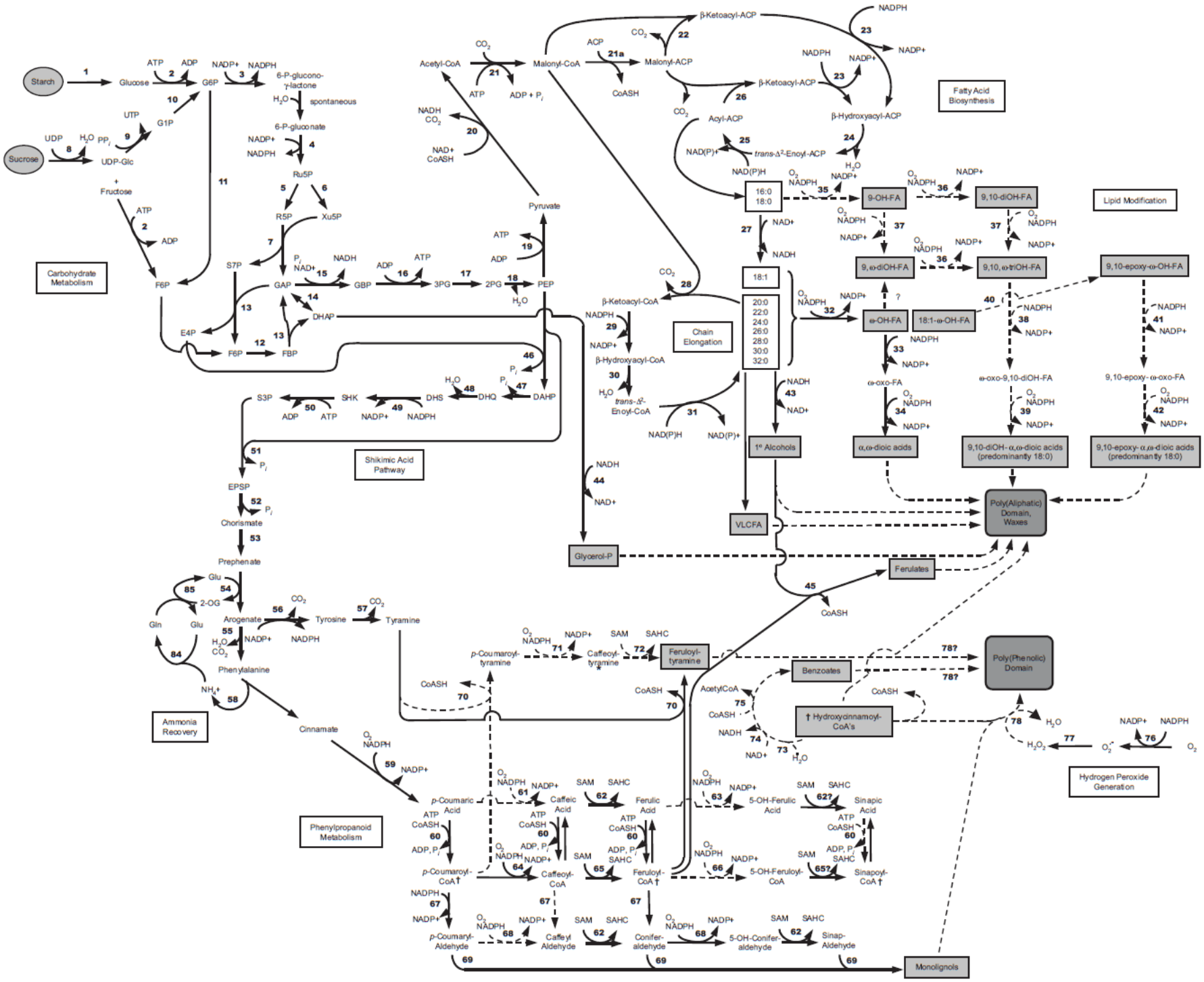
Starch
Glucose
G6P
6-P-gluconolactone
6-P-gluconate
Ru5P
R5P
Xu5P
S7P
E4P
F6P
FBP
GAP
DHAP
GBP
3PG
2PG
PEP
Pyruvate
G1P
UDP-Glc
Sucrose
Fructose
Carbohydrate Metabolism
Acetyl-CoA
Malonyl-CoA
Malonyl-ACP
β-Ketoacyl-ACP
β-Hydroxyacyl-ACP
Acyl-ACP
trans-Δ²-Enoyl-ACP
Fatty Acid Biosynthesis
16:0
18:0
9-OH-FA
9,10-diOH-FA
9,ω-diOH-FA
9,10,ω-triOH-FA
ω-OH-FA
18:1-ω-OH-FA
9,10-epoxy-ω-OH-FA
Lipid Modification
18:1
20:0
22:0
24:0
26:0
28:0
30:0
32:0
β-Ketoacyl-CoA
β-Hydroxyacyl-CoA
trans-Δ²-Enoyl-CoA
Chain Elongation
1° Alcohols
ω-oxo-FA
ω-oxo-9,10-diOH-FA
9,10-epoxy-ω-oxo-FA
α,ω-dioic acids
9,10-diOH-α,ω-dioic acids (predominantly 18:0)
9,10-epoxy-α,ω-dioic acids (predominantly 18:0)
Poly(Aliphatic) Domain, Waxes
VLCFA
Glycerol-P
Ferulates
CoASH
S3P
SHK
DHS
DHQ
DAHP
Shikimic Acid Pathway
EPSP
Chorismate
Prephenate
Arogenate
Tyrosine
Tyramine
Phenylalanine
Cinnamate
Glu
2-OG
Gln
NH₄⁺
Ammonia Recovery
p-Coumaric Acid
Caffeic Acid
Ferulic Acid
5-OH-Ferulic Acid
Sinapic Acid
p-Coumaroyl-CoA†
Caffeoyl-CoA
Feruloyl-CoA†
5-OH-Feruloyl-CoA
Sinapoyl-CoA†
p-Coumaryl-Aldehyde
Caffeyl Aldehyde
Coniferaldehyde
5-OH-Coniferaldehyde
Sinapaldehyde
Monolignols
Phenylpropanoid Metabolism
p-Coumaroyl-tyramine
Caffeoyl-tyramine*
Feruloyl-tyramine
Benzoates
† Hydroxycinnamoyl-CoA's
AcetylCoA
Poly(Phenolic) Domain
Hydrogen Peroxide Generation
H₂O₂
O₂⁻
O₂
SAM
SAHC
ATP
ADP
ADP + Pᵢ
NADPH
NADP+
NADH
NAD+
NAD(P)H
NAD(P)+
CO₂
H₂O
Pᵢ
PPᵢ
UTP
UDP
ACP
CoASH

**Fig. 7.** General biosynthetic scheme for the precursors of suberin polyaliphatic and polyaromatic domains starting with starch and sucrose (the two main sources of biosynthetically available carbon in plants). The layout represents a composite of knowledge derived from a number of different plant species (principally potato and maize). The enzymes indicated are as follows (enzyme names followed by an * are hypothetical): **1**, starch degrading enzymes; **2**, hexokinase; **3**, glucose-6-phosphate dehydrogenase; **4**, phosphogluconate dehydrogenase; **5**, ribulose-5-phosphate isomerase; **6**, ribulose-5-phosphate epimerase; **7**, transketolase; **8**, sucrose synthase; **9**, UDP-glucose pyrophosphorylase; **10**, glucose-6-phosphate isomerase; **11**, phopshoglucose isomerase; **12**, pyrophosphate – fructose-6-phosphate 1-phosphorylase; **13**, aldolase; **14**, triose phosphate isomerase; **15**, glyceraldehyde-3-phosphate dehydrogenase; **16**, phosphoglycerate kinase; **17**, phosphoglyceromutase; **18**, enolase; **19**, pyruvate kinase; **20**, pyruvate dehydrogenase; **21**, acetyl-CoA carboxylase; **21a**, malonyl-CoA:ACP transacylase; **22**, β-ketoacyl-ACP synthetase III; **23**, β-ketoacyl- ACP reductase; **24**, β-hydroxyacyl-ACP dehydratase; **25**, enoyl-ACP reductase; **26**, β-ketoacyl-ACP synthetase I, II; **27**, stearoyl- ACP Δ9-desaturase; **28**, β-ketoacyl-CoA synthase III; **29**, β-ketoacyl-CoA reductase; **30**, β-hydroxyacyl-CoA dehydratase; **31**, enoyl- CoA reductase; **32**, fatty acyl-ω-hydroxylase; **33**, ω-hydroxyacid dehydrogenase; **34**, ω-oxoacid dehydrogenase; **35**, fatty acyl-9- hydroxylase*; **36**, 9(ω)-hydroxy fatty acyl-10-hydroxylase*; **37**, 9(10)-hydroxy fatty acyl-ω-hydroxylase*; **38**, 9,10,ω-trihydroxyacid dehydrogenase*; **39**, 9,10-dihydroxy-ω-oxoacid dehydrogenase*; **40**, ω-hydroxyacid-9,10-epoxide synthase*; **41**, 9,10-epoxy-ω-hydroxyacid dehydrogenase*; **42**, 9,10-epoxy-ω-oxoacid dehydrogenase*; **43**, reductases*; **44**, glycerol-3-phosphate dehydrogenase; **45**, hydroxycinnamoyl-CoA:1-alkanol hydroxycinnamoyl transferase; **46**, DAHP synthase; **47**, dehydroquinate synthase; **48**, 3- dehydroquinate dehydratase; **49**, 3-dehydroshikimate reductase; **50**, shikimate kinase; **51**, EPSP synthase; **52**, chorismate synthase; **53**, chorismate mutase; **54**, prephenate aminotransferase; **55**, arogenate dehydratase; **56**, arogenate dehydrogenase; **57**, tyrosine decarboxylase; **58**, phenylalanine ammonia-lyase; **59**, cinnamate-4-hydroxylase; **60**, 4-coumaroyl-CoA ligase; **61**, p-coumaric acid 3-hydroxylase; **62**, caffeic acid 3-O-methyltransferase; **63**, ferulic acid 5-hydroxylase; **64**, p-coumaroyl-CoA-3- hydroxylase; **65**, caffeoyl-CoA-3-O-methyltransferase; **66**, hydroxycinnamoyl-CoA-5-hydroxylase*; **67**, cinnamoyl-CoA oxidoreductase; **68**, hydroxycinnamaldehyde hydroxylase*; **69**, coniferyl alcohol dehydrogenase; **70**, hydroxycinnamoyl-CoA:tyramine hydroxycinnamoyltransferase; **71**, p-coumaroyltyramine-3-hydroxylase*; **72**, caffeoyltyramine-O-methyltransferase*; **73**, hydroxycinnamoyl-CoA-7-hydroxylase*; **74**, (7-hydroxy)-hydroxycinnamoyl-CoA reductase*; **75**, thiolase*; **76**, NAD(P)H-dependent oxidase; **77**, superoxide dismutase or spontaneous; **78**, peroxidase; **84**, glutamine synthase; **85**, glutamine:2-oxoglutarate aminotransferase. Reactions denoted by solid lines are known, while those denoted by broken lines are hypothetical or assumed. Shaded boxes denote known precursors incorporated into the suberin polyaliphatic and polyaromatic domains (taken from Bernards 2002).

### 3.2. Molecular studies on suberin biosynthesis and cork development

Cork composition and cellular structure are quite well established, but the molecular mechanisms underlying its biosynthesis are poorly understood. Soler et al. (2007) performed the first genome-wide approach on cork oak bark, providing a list of candidate genes for cork biosynthesis and regulation. This list includes genes for fatty acid elongase complexes, ATP-binding cassete transporters, acyltransferases, phenylpropanoid pathway enzymes, several transcription factors and regulatory proteins, among others, More recently, other comprehensive

study generate a list of candidate genes for potato periderm and suberin biosynthesis (Soler et al. 2011). In potato, genes for fatty acid elongation, acyltransferase, phenylpropanoid pathway enzymes and regulatory proteins were also identified. But the most abundant category includes genes for stress response and defence. Most of these candidate genes remain to be functionally characterized. Up to date, only few studies describe mutants from *Arabidopsis* and potato with altered suberin. This is the case of two knockout mutants of *Arabidopsis* GPAT5 (*gpat5-1* and *gpat5-2*), encoding a protein with acyl-*CoA*: glycerol-3-P acyltransferase activity (Beisson et al. 2007). *gpat5* mutants show changes on aliphatic suberin composition in seed coats and roots, with consequences on seed coat permeability and seedlings tolerance to salt stress. Hoofer et al. (2008) characterized knockout mutants of the cytrochrome P450 fatty acid ω-hydroxilase CPY86A1 from *Arabidopsis*. Authors concluded that the corresponding *hydroxylase of roots suberized tissues (horst)* mutant exhibits a highly reduced aliphatic root suberin as well as an altered monomer composition. Other study, also performed in *Arabidopsis*, describes mutants of b-ketoacyl-CoA-synthases (KCS) that catalyses the elongation of C20 acyl chain suberin percursors (Lee et al. 2009). These authors observed that the *kcs20 kcs2/daisy-1 double* mutant shows a significant reduction of C22 and C24 very-long-chain fatty acids derivatives in root suberin whereas C20 derivatives were accumulated. Domergue et al. (2010) characterized three mutants of fatty acyl-coenzyme A reductase (FAR) from *Arabidopsis* (*far1, far4 and far5*) that show affected primary alcohol formation in root, seed-coat and wound-induced leaf tissue. In potato, two other studies have reported the molecular and physiological function of *StKCS6* and *CYP86A-33* genes. In mutants of *StKCS6* authors observed a reduction of compounds with chain lengths of C28 and higher (Serra et al. 2009a). Mutants of *CYP86A-33* show a strong decrease of the ω-functionalized monomers and distorted suberin lamellae (Serra et al. 2009b). The knowledge about the molecular mechanisms underlying the synthesis of aromatic suberin is even scarcer. The first gene identified as being required for the synthesis of aromatic suberin components, the *AFST/HHT*, encodes a feruloyl transferase member of the BAHD superfamily (Gou et al. 2009, Molina et al. 2009). Knockout mutants of this gene show a reduced quantity of ferulate in suberin, thus confirming the essential role of these enzymes in the incorporation of ferulate into suberin. In potato, Serra et al. (2010) have also observed the same results in mutants of its orthologous, the *FHT*.

In the next years it is expected that the information resulting from recent Portuguese initiatives on systematic cork oak transcriptome and genome analyses, will enable significant progresses on cork biosynthesis research in this species.

## 4. Transcriptional regulation of secondary growth

The secondary growth of woody stems is a dynamic process that includes numerous developmental mechanisms and responds to physical and environmental cues. With the advances in genomic technologies, applied to forest trees, several mechanisms have been described as

regulators of secondary growth. Together with phytohormones and information molecules of the cell wall, the transcriptional regulation is among the mechanisms with central roles in the control of secondary growth (Groover and Robischon 2006). Most of what is known about transcriptional regulation of secondary growth focuses on wood formation and lignin biosynthesis in particular. In recent years, several transcription factors from different gene families and different tree species have been identified and characterized. The MYB family comprises the majority of lignin/secondary growth activators (Zhao and Dixon 2011). This is the case of the EgMYB2 from *Eucalyptus gunnii* whose overexpression in tobacco plants caused a dramatic increase in secondary cell wall thickness and an alteration of the lignin profiles (Goicoechea et al. 2005). The PtMYB1 and PtMYB8 from *Pinus taeda* also showed to be involved in phenylpropanoid metabolism and secondary cell wall biogenesis when constitutively overexpressed in *Picea* (Bomal et al. 2008). Other example is the overexpression of the two poplar MYB transcription factors, the PtrMYB3 and PtrMYB20 that activated the biosynthetic pathways of cellulose, xylan and lignin (McCarthy et al. 2010) in *Arabidopsis*. But, MYB TFs can also act as repressors as is the case of EgMYB1 TF from *E. gunnii,* which binds to *EgCCR* and *EgCAD2 cis*-regulatory regions in vitro and acts as a transcriptional repressor of these genes in vivo (Legay et al. 2007). Other example is the PttMYB21a from *Populus tremula x tremuloides*, which is a homolog of *Arabidopsis* AtMYB52 and negatively affects the expression of *CCoAOMT* gene when expressed in hybrid aspen plants in the antisense orientation. Moreover, the levels of acid soluble lignin were higher in the transgenic lines than in wild-type plants (Karpinska et al. 2004). TFs from other families have also been associated to secondary growth, namely, members of class-I KNOX, which are well characterized for their involvement in regulating shoot apical meristem (SAM) (Du and Groover 2010). For instance, the *ARBORKNOX1*, *a Populus* orthologous of the *Arabidopsis SHOOTMERISTEMLESS* gene showed to be expressed not only in SAM but also in the cambial zone of *Populus.* Additionally its overexpression in *Populus* resulted in a delay of secondary growth and stems with highly reduced phloem fibers and secondary xylem as well as an altered expression of numerous genes involved in cell wall biosynthesis and lignification (Groover et al. 2006). More recently, the *ARBORKNOX2* gene, a *Populus* orthologous of the *Arabidopsis BREVIPEDICELLUS* showed to influence terminal cell differentiation and cell wall properties during secondary growth. It was also observed a negative association of its expression with lignin and cellulose content and thereby with wood properties (Du et al. 2009). Other class of TFs that has been described as master regulators of secondary growth is the NAC domain TFs, such as the PtrWND2B and PtrWND6B from *Populus trichocarpa* that are functional orthologous of the *Arabidopsis* SND1, and whose overexpression leads to ectopic deposition of secondary walls in *Arabidopsis*. Furthermore, they are also able to activate the promoter activities of several wood-associate TFs and wood biosynthetic genes (Zhong et al. 2011). Numerous TFs involved in specific processes of secondary growth have already been functionally characterized, however, a comprehensive understanding of how these genes act together to affect secondary growth and development is still missing (Liu et al. 2013).

The knowledge about the transcriptional regulation underlying secondary growth derived from cork cambium is much more limited than the knowledge on vascular cambium. In the first genomic approach to suberin biosynthesis and cork differentiation, Soler et al. (2007) identified some regulatory genes with putative functions in meristem identity and cork differentiation. Among these genes, the authors identified some TFs including MYB, No-Apical-Meristem (NAM) and WRKY. However, to our knowledge, none of these genes was functionally characterized in cork oak. But, in other species, as mentioned above, MYB TFs have been associated to the regulatory networks controlling development, metabolism and (a)biotic stress response (Dubos et al. 2010). NAM TFs are proteins belonging to NAC family due to the presence of a NAC DNA-binding domain. Members of NAC family have been implicated in diverse processes of development, defence and abiotic stress response (Olsen et al. 2005). WRKY TFs comprise a large family that shows a high binding-affinity to W-boxes and has been suggested as key factors in plant immune responses (Eulgem and Somssich 2007) and also in response to abiotic stress, leaf senescence, trichomes development or in regulation of biosynthetic pathways (Chen et al. 2012). Recently, Miguel et al. (2011) reported the cloning of a *SHORT-ROOT (SHR)* transcription factor from GRAS family with putative function in regulation of phellogen.

## 5. MYB transcription factors superfamily

MYB (**my**elo**b**lastosis) superfamily of proteins is large, functionally diverse and is found in nearly all eukaryotes (Dubos et al. 2010). The first identified MYB gene was the oncogene *v-MYB* from avian myeloblastosis virus (AMV) and afterwards other related genes – *c-MYB, A-MYB* and *B-MYB* were found in many vertebrates (Stracke et al. 2001). Transcription factors from MYB superfamily are characterized by having a highly conserved DNA binding motif, the MYB domain, located in the N-terminus of the protein (Du et al. 2009). This MYB domain typically consists of one to four imperfect repeats (R) of about 52 amino acids, each forming three α-helices with the second and third helices building a helix-turn-helix (HLH) structure when bound to DNA (Dubos et al. 2010). Other characteristic of a MYB repeat is the presence of three regularly spaced tryptophan residues forming a hydrophobic core in the HLH structure that stabilizes the structure of the DNA binding domain (Du et al. 2009). MYB TFs superfamily can be classified into four major families depending on the number of MYB adjacent repeats (Katiyar et al. 2012). The three repeats on *c-MYB* are referred as R1, R2 and R3 and repeats from other MYB-proteins are categorized according to their similarity to R1, R2 or R3 of *c-MYB* (Stracke et al 2001). Proteins with single or a partial MYB repeat are designated as MYB-related; with two repeats are named as R2R3-MYB, with three repeats as R1R2R3-MYB and with four repeats as 4R-MYB, which is the smallest group whose members contain four R1/R2 repeats (Li et al. 2006, Dubos et al. 2010).

### 5.1. R2R3-MYB family

In plants, the most common family of MYB TFs and also the most characterized is the R2R3-MYB family (Jin and Martin 1999). Contrarily to the conserved MYB domain, the C-terminus region of MYB proteins is highly variable and usually functions as either an activator or a repressor (Prouse and Campbell 2012). However, a number of conserved amino acid motifs were found in the region outside the MYB domain. Based on sequence similarities of both C-terminal motifs and MYB domain, the R2R3-MYB family was divided into 22 subgroups in *A. thaliana* (Kranz et al. 1998, Stracke et al. 2001). But, comparative phylogenetic analysis have identified new R2R3-MYB subgroups in other plant species for which there are no representatives in *A. thaliana* (e.g. poplar grapevine and maize) (Dubos et al. 2010, Du et al. 2012).

The R2R3-MYB family has been widely characterized mainly in Arabidopsis, but with the increasing availability of plant genome sequences, the knowledge about this TFs family is being extended (Du et al. 2012). Studies have demonstrated that R2R3-MYB TFs regulate a myriad of plant-specific processes. These processes include regulation of plant primary and secondary metabolism as in the case of PtMYB1, PtMYB8 and PtMYB4 from *P. taeda* that are suggested to be involved in regulation of lignin biosynthesis (Bomal et al. 2008, Patzlaff et al. 2003); or MYB110a that control anthocyanin production in petals of an *Actinidia* hybrid population (Fraser et al. 2013); or AtMYB41 that showed to regulate primary metabolism in *Arabidopis* (Lippold et al. 2009). Some R2R3-MYB TFs showed to be involved in the control of cell fate and identity, namely AtMYB66/WER, AtMYB23 and AtMYB0/GL1 that regulate the epidermal cell fate determination in *Arabidopsis* (Tominaga-Wada et al. 2012); or AtMYB17 described as a putative regulator of early inflorescence development and seed germination (Zhang et al. 2009). Some developmental processes are also regulated by R2R3-MYB TFs, for instance, members of subgroup-14 such as AtMYB37/RAX1, AtMYB38/RAX2 and AtMYB84/RAX3, which are partially redundant regulators of axillary meristems at different developmental times (Müller et al. 2006). Finally, R2R3-MYB TFs are also described as players in the regulatory networks of responses to (a)biotic stresses and in signal transduction pathways of phytohormones. This is the case of AtMYB96 that regulates drought response and pathogen resistance by integrating abscisic acid signal in *Arabidopsis* (Seo et al. 2009, Seo et al. 2010); or the AtMYB68 whose expression is modulated by temperature and its loss function mutant shows a reduced ability of growing at high temperatures (Feng et al. 2004).

## Main objectives of this PhD thesis

The overall objective of this PhD thesis is to contribute for a better understanding of the transcriptional regulatory network underlying cork biosynthesis, which is very limited. Increasing the knowledge on these molecular mechanisms will be a step forward to develop new strategies to better control cork properties in the future. Given the crucial role of transcription factors in

controlling transcriptome, the research of this thesis was focused on an R2R3-MYB transcription factor, the QsMYB1. *QsMYB1* was previously identified as a candidate gene involved in cork differentiation and development. The specific objectives are:

- To perform the molecular characterization of *QsMYB1* by determining the complete gene structure and by analyzing *QsMYB1* expression in a wide range of organs and tissues from cork oak (Chapter II).

- To assess the effects of abiotic stresses on *QsMYB1* expression profile by exposing cork oak plants to high temperature or drought stresses (two common environmental conditions during the cork growth period). (Chapter III).

- To evaluate if phytohormones (important regulators of plant growth and development as well as of response to a(biotic) stresses) regulate *QsMYB1* expression when exogenously applied to cork oak plants (Chapter IV).

- To perform the functional characterization of QsMYB1 through a reverse genetic approach in a heterologous system (Chapter V).

At the end of this PhD thesis it is expected to have new insights into the putative function(s) of QsMYB1 within the regulatory network of cork development.

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## CHAPTER II

# Molecular characterization of *Quercus suber* MYB1, a transcription factor up-regulated in cork tissues

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## Abstract

The molecular processes associated with cork development in *Quercus suber* L. are poorly understood. A previous molecular approach identified a list of genes potentially important for cork formation and differentiation, providing new basis for further molecular studies. This report is the first molecular characterization of one of these candidate genes, *QsMYB1*, coding for an R2R3-MYB transcription factor. The *R2R3-MYB* gene family has been described as associated with the phenylpropanoid and lignin pathways, both involved in cork biosynthesis. The results showed that the expression of *QsMYB1* is putatively mediated by an alternative splicing (AS) mechanism that originates two different transcripts (*QsMYB1.1* and *QsMYB1.2*), differing only in the 5'-untranslated region, due to retention of the first intron in one of the variants. Moreover, within the retained intron a simple sequence repeat (SSR) was identified. The upstream regulatory region of *QsMYB1* was extended by a genome walking approach, which allowed the identification of the putative gene promoter region. The relative expression pattern of *QsMYB1* transcripts determined by reverse-transcription quantitative polymerase chain reaction (RT-qPCR) revealed that both transcripts were up-regulated in cork tissues; the detected expression was several folds higher in newly formed cork harvested from trees producing virgin, second or reproduction cork when compared with wood. Moreover, the expression analysis of *QsMYB1* in several *Q. suber* organs showed a very low expression in young branches and roots, whereas in leaves, immature acorns or male flowers no expression was detected. These preliminary results suggest that *QsMYB1* may be effectively related to secondary growth and in particular to the cork biosynthesis process, having a possible alternative splicing mechanism associated with its regulatory function


***Keywords*:** Alternative splicing, cork biosynthesis, cork oak, phellogen, R2R3-MYB.

***Abbreviations:*** AS, alternative splicing; CB, cork branch; EST, expressed sequence tag; IA, immature acorns; L, leaves; MF, male flowers; nd, not detected; RACE, rapid amplification cDNA ends; RC, reproduction cork; Rt, roots; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; SC, second cork; SSR, simple sequence repeat; TF, transcription factor; VC, virgin cork; W, wood; YB1, one-year-old young branches.

## 1. Introduction

Cork (or phellem) formation is a secondary growth derived process that results from the activity of the phellogen or cork cambium. Cork oak (*Quercus suber* L.) has the unique ability of developing a phellogen that is active during tree's life, producing every year, a thick layer of suberized cork cells that accumulate in the outer bark of the tree in the form of annual rings. When the initial phellogen is destroyed, e.g., by removal of the cork layer or as consequence of a drought/high temperature period, a traumatic phellogen differentiates in the outer phloem. The successive formation of traumatic phellogens following removal of cork at periodic intervals (e.g., every 9-10 years) allows for the exploitation of the cork oak on a sustainable basis (Graça and Pereira 2004).

Cork cellular structure, chemical composition, and physical and mechanical properties have been well described (Silva et al. 2005). The properties of cork are related to its chemical composition (suberin, lignin, polysaccharides and extractives) and to the molecular structure of its components. Although the presence of these components may show some variations in different types of cork (Pereira 1988), it is clear that cork is marked by a high deposition of suberin, the main component on cork cell walls of *Q. suber* periderm. Suberin is a lipid polyester that offers protection against dehydration and pathogens. Molecular studies on suberin biosynthesis have focused mainly on the use of suberin related mutants of *Arabidopsis thaliana:* the *elongation defective1*, a pleiotropic mutant showing ectopic suberin deposition (Cheng et al. 2000), two knockout mutants for the acyl-CoA: glycerol-3-P acyltransferase 5 gene – *GPAT5* (*gpat5-1* and *gpat5-2*) with altered suberin (Beisson et al. 2007), the *horst* mutant with reduced amount of aliphatic suberin in roots (Hoofer et al. 2008) and double mutants for b-ketoacyl-CoA-synthases (KCS, *kcs20 kcs2/daisy-1*) showing reductions of C22 and C24 very-long-chain derivatives in root suberin (Lee et al. 2009). Two studies using the model plant for suberin biosynthesis, the potato, have reported the molecular and physiological function of *StKCS6* and *CYP86A-33* genes in the biosynthesis of suberin and wax compounds of potato tuber periderm (Serra et al. 2009a; b). Another recent work has showed that the down-regulation of a feruloyl transferase (FHT) involved in the biosynthesis of suberin, resulted in altered anatomy, sealing properties and maturation of the tuber periderm (Serra et al. 2010).

The biosynthesis of suberin aromatic components and the lignin polymers derive both from the general phenylpropanoid pathway and share the same basic reactions (Rahantamalala et al. 2010). Since global xylem transcript profiling has already been reported for *Arabidopsis* and several tree species (Kirst et al., 2004; Andersson-Gunneras et al. 2006), a significant progress has been made in understanding the biosynthetic pathway of lignin (Vanholme et al. 2010). Several *MYB* genes have been demonstrated to affect the expression of lignin biosynthetic genes when overexpressed (Patzlaff et al. 2003, Goicoechea et al. 2005, Legay et al. 2007, Zhou et al. 2009). In spite of the reports mentioned above, studies focusing on the processes underlying cork biosynthesis and differentiation are still scarce. The tissue availability (restricted to a narrow time

frame) and the engagement of cork cells in programmed cell death processes are within the main reasons. However, a very recent proteomic analysis made by Ricardo et al. (2011) identified 54 proteins specifically associated with cork formation in cork oak stem. Previously, a genomic approach to cork formation had already been reported in *Q. suber*, where a collection of candidate genes with putative functions in meristem identity and cork differentiation was obtained (Soler et al. 2007). The up-regulated genes identified in cork tissues included genes for the synthesis, transport and polymerization of suberin monomers as well as regulatory genes, namely, transcription factors such as MYB-like proteins that comprise a large group of transcription factors involved in plant development and tissue differentiation. This group includes proteins with a DNA binding motif, the MYB domain, located in the N-terminus domain of the protein. In animals, the conserved domain of MYB proteins contains three imperfect repeats of 50-53 amino acids (R1, R2, and R3), while most plant MYB proteins are of the R2R3 type and are thought to be predominantly involved in plant specific regulatory processes (Dubos et al. 2010). Members of the R2R3-MYB transcription factors family have been implicated as regulators of phenylpropanoid and lignin metabolism (Tamagnome et al. 1998, Borevitz et al. 2000, Bomal et al. 2008), pattern formation and differentiation of primary and secondary vascular tissues (Glover et al. 1998) and abiotic (Abe et al. 1997) and biotic stress responses (Mengiste et al. 2003). Nevertheless, the functions of most of these R2R3 MYB proteins remain unknown. Based on this, we selected an R2R3-MYB transcription factor, that we named *QsMYB1*, from the list of genes identified by Soler et al. (2007), considering that a deep knowledge on this gene would be an important contribution to the understanding of the molecular processes behind cork biosynthesis. We first determined the *QsMYB1* gene organization in terms of introns and exons structure and identified the putative promoter region. By sequence analysis we also found that an alternative splicing mechanism is possibly associated to *QsMYB1* expression. Further, we analysed the expression pattern of the two identified transcripts in different tissues and organs of *Q. suber* and along cork development to obtain clues for the putative regulatory function of QsMYB1.

## 2. Materials and methods

### 2.1. Plant material

Three year old cork oak plants grown in 400 $cm^3$ containers with peat-vermiculite (3:1, v/v) were used as source of leaves (between $1^{st}$ and $2^{nd}$ internodes) and young side branches (one year old). Cork tissues were harvested from field-grown cork trees at Herdade da Boavista (Beja, Portugal) during the growing season (July). External bark (cork bark) was removed and, using sterile scalpels, the newly formed phellem cells were harvested. Phellem samples were collected according to the distinct cork extractions that occur at a 9-10 year interval during cork oak's life (virgin, second and reproduction cork). Samples of phellem and wood were also harvested from

branches of field-grown trees at Herdade da Boavista (Beja, Portugal) in May. The external bark of the branch was firstly removed and phellem and wood samples were collected as described above. Immature acorns and male flowers were collected from field-grown trees at Ferreira do Alentejo (Beja, Portugal) during spring season (April). Root samples, comprising only the differentiation zone, were harvested from acorns germinated in sand during approximately 15 days. After sampling, organs and tissues were frozen in liquid nitrogen and stored at -80 ºC until further use.

**2.2. RNA extraction and cDNA synthesis**

Total RNA was extracted from cork tissue (virgin, second and reproduction), young branches, immature acorns and leaves using the method described by Chang et al. (1993) with modifications done by Soler et al. (2008). At the end, residual DNA was removed from RNA samples using the RNase Free DNase Set (Qiagen) and further purified following the Cleanup Protocol from the RNeasy Plant Mini Kit (Qiagen). Chang et al. (1993) method was also tested with the other samples (male flowers, root, wood and cork branch); however, RNA quality did not meet the requirements to proceed. Thus, the total RNA from male flowers and root was isolated with the RNaqueous4PCR Kit (Ambion) whereas for wood and cork branch samples, the Spectrum Plant Total RNA kit (Sigma-Aldrich) was used. In both procedures, protocols were followed according to manufacturer's instructions which included a DNase treatment. A pool of 3 biological samples was used in each RNA extraction. RNA concentration and purity were assessed with a NanoDrop® ND-1000 spectrophotometer (Thermo scientific) and the integrity was verified on a 1% agarose gel in 1x TBE/DEPC water. For cDNA synthesis only RNA samples with ratios of $A_{260/230}$ in the range of 2 - 2.2 and $A_{260/280}$ in the range of 1.8 - 2.2 were used. One microgram of total RNA was reverse transcribed using the QuantiTect Reverse Transcription kit (Qiagen), which includes an additional genomic DNA elimination step and uses a mix of oligo(dT) and random hexamer primers. Synthesis of cDNA was performed in duplicate for every RNA samples.

**2.3. DNA extraction**

DNA was isolated from leaves of the three year old potted plants according to the Doyle and Doyle (1990) procedure. A pool of 3 biological samples was used for DNA extraction. The purity and quantity of the DNA samples was analysed in a spectrophotometer and integrity was checked by electrophoresis on a 1% agarose gel.

**2.4. Characterization of exon/intron structure of QsMYB1**

Based on the expressed sequence tag (EST) from *QsMYB1* (Accession number EE743680), previously reported in Soler et al. (2007), a pair of primers was designed, QsR2R3MYBFwd (5'CCTTGCTCTCCTATTCTGGC3') and QsR2R3MYBRev (5'ATATCATTATCTGTTCTTCCTG3') to

amplify the complete EST. PCR reactions mixtures (25 µL) contained 200 µM dNTPs, 0.2 µM of each primer, 0.05 U / µL of DreamTaq (Fermentas) and 50 ng of genomic DNA or 2 µL of cDNA from second cork. PCR was carried out as follow: initial denaturation step of 94 °C for 4 min, followed by 34 cycles of denaturation (95 °C for 45 s), annealing (57 °C for 45 s) and elongation (72 °C for 30 s) with a final extension step at 72 °C for 10 min. PCR products were cloned in the pCR® 2.1 TOPO® vector (Invitrogen) and sequenced.

### 2.5. Genome walking and Rapid Amplification of cDNA Ends (RACE)

A genome walking approach was performed in order to amplify the genomic sequence of *QsMYB1*, using the Universal GenomeWalker™ kit (Clontech) to extend both 3' and 5' ends. Based on the already known DNA sequence, two sets of gene-specific primers (GSP) were designed, the outer primers GSP1F (5'TCAGACCCTGCAAAAGTTAGAGTTTTGTGTGC3'), GSP1R (5'AGCTCTCCCCATGGTGTTGCTTTGAGCTTGA3') and the nested ones GSP2F (5'TATCATTTGGTTGGCATGAACAGGTGGTCTA3') and GSP2R (5'AGCCTATAGCTTCTCCCTCTGAAACCCAAA3'). Genomic DNA from *Q.suber* was digested with four blunt end cutting enzymes (*Eco*RV, *Pvu*II, *Stu*I and *Sma*I) to construct the DNA libraries which were further purified and ligated to the GenomeWalker adaptor (provided in the kit). A primary PCR was performed with the outer adaptor primer (AP1) and the corresponding outer gene-specific primer to walk either in the 5'end direction (GSP1R) or in the 3' end direction (GSP1F). The product of this primary PCR was then diluted (1:50) and used as template for a second walk using the nested adaptor primer (AP2) and one of the gene-specific primers (GSP2F or GSP2R). The PCR reactions were performed with the Phusion High Fidelity DNA Polymerase (Finnzymes), cloned into the pJET1.2 vector (Fermentas) and sequenced.

The cDNA ends of QsMYB1 were amplified using the 5'/3' RACE, 2nd generation kit (Roche Diagnostics GmbH) according to manufacturer's instructions with minor modifications. For 5' RACE, 2.5 µg of second cork RNA was reverse transcribed with the gene specific primer SP1 (5'AGCTCTCCCCATGGTGTTGCTTTGAGCTTGA3') and further amplified with SP2 (5'TAGCTTCTCCCTATGGTTATTAGG3') and SP3 (5'CCTCTGAAACCCAAATAAGAATAT3') primers to specifically amplify the 5'ends of *QsMYB1.1* and *QsMYB1.2*, respectively. One µg of second cork RNA was used for the 3' RACE using the primer SP4 (5'GGGCTAGAGTTCTTATGTGGGGAGGACATGG3'). The PCR cycling conditions were the same for all reactions, as follows: initial denaturation step at 98 ºC for 30 s followed by 35 cycles of 98 ºC (10 s), 54 ºC / 57 ºC / 60 ºC (SP2/SP3/SP4) and 72 ºC for 30 s each. The cDNA fragments were amplified with the Phusion High Fidelity DNA Polymerase (Finnzymes), cloned into the pJET1.2 vector (Fermentas) and sequenced.

### 2.6. Sequence analysis

Sequence manipulation and analysis was performed using the BioEdit graphic interface (Hall 1999) and CLUSTALW algorithm (Thompson et al. 1994). BLAST Network Service (http://www.ncbi.nlm.nih.org) was used for search against the GenBank database. Promoter analysis was performed using the PlantPan interface (http://PlantPAN.mbc.nctu.edu.tw) (Chang et al. 2008). Three *QsMYB1* sequences were deposited in GeneBank: the *QsMYB1* genomic sequence including the putative promoter region (accession number JN003628) and the two splicing variants, *QsMYB1.1* (accession number JF970262) and *QsMYB1.2* (accession number JF970263).

### 2.7. Analysis of transcript abundance by RT-qPCR

To specifically amplify both *QsMYB1* transcripts, the above QsR2R3MYB (Forward) primer was used together with one of the following reverse oligonucleotides: BND (5'TAGCTTCTCCCTATGGTTATTAGG3') to amplify the *QsMYB1.1* transcript (amplicon of 118 bp) or the SSR (5'CCTCTGAAACCCAAATAAGAATAT3') for the amplification of *QsMYB1.2* transcript (amplicon of 195 bp). The PCR mixture (20 µL) included 1X iQ™SYBR® Green Supermix (Bio-Rad Laboratories), 0.2 µM of each primer and 2 µL of the previously synthesized cDNA. No template controls were also prepared by omission of template and addition of water instead. Absence of genomic DNA contamination was also confirmed by RT-qPCR using a pair of primers spanning an intron in the coding region: Ex1 (5′GGGAGAGCTCCATGTTGTGAC3′) designed in exon 1 and Ex2 (5′CAAGAGTTGCCGCCTTAGATGGTTAA3′) in exon 2 using an annealing temperature of 58℃ and with an expected amplicon of 326 bp in case of DNA contamination. Three technical replicates were performed. Real time PCR runs were carried out in 96-well optical plates. The following PCR profile was used: an initial incubation at 95°C for 3 min, followed by 40 cycles with 10 s at 95 ºC, 15 s at 57 °C / 60 ºC (*SSRRev* / *BNDRev*) and 10 s at 72 °C with a single fluorescent reading taken at the end of each cycle. To distinguish specific from nonspecific products and primer dimers, a melting curve was obtained immediately after amplification. PCR and melting products were detected in real time with an iCycler iQ5 Instrument (Bio-Rad Laboratories). The threshold cycles ($C_T$) were calculated by the optical interface iQ5 software (Bio-Rad Laboratories). Three reference genes, previously described (Soler et al. 2008), were tested: β- tubulin (β-*TUB*), actin (*ACT*) and polymerase elongation factor-1α (*EF*-1α). PCR conditions and cycling parameters were the same as before with an annealing temperature of 60 °C. According to NormFinder (Andersen et al. 2004) analysis, the *ACT* and *EF*-1α genes were chosen as the most stable ones. The geometric mean of the Ct values of both genes was used to normalize samples. Relative abundances of *QsMYB1* transcripts were calculated by the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Two sets of samples were considered: the group of tissues and the group of organs in which wood or root sample was chosen as calibrator, respectively.

## 3. Results

### 3.1. QsMYB1 exon/intron structure

The amplification of cDNA using the pair of primers QsR2R3MYBFwd / QsR2R3MYBRev designed in the exon E0 and in the exon E3, respectively, revealed consistently the presence of two transcripts, differing only in 86 bp in length, in several organs and tissues of *Q. suber*. On the other hand, genomic DNA amplification, with the same pair of primers, resulted only in one fragment of 851 bp (data not shown), indicating that an AS mechanism was possibly mediating the *QsMYB1* gene expression.

The extension of about 1000 bp of the genomic sequence to both 5' and 3' ends was possible by genome walking, which allowed the gene structure characterization (Fig. 1). The alignment of the genomic sequence with the available cDNA sequences indicated a 5'-UTR with 2 exons (E0 – 246 bp; E0 – 42 bp) and one intron (I0 – 85 bp), exhibiting a canonical GT / AG splicing motif. The open reading frame is composed of 3 exons (E1 – 136 bp; E2 – 130 bp and E3 – 772 bp) and 2 introns (I1 – 151 bp; I2 – 151 bp). The first intron has a canonical GT / AG exon-intron junction whereas the second one shows a non-canonical terminal AG / TA dinucleotide. Both introns are fully spliced after transcription. The 3'-UTR has 100 bp. BLASTP performed against GenBank database, revealed that the putative protein sequence had 87% of similarity and 92% of identity with the DNA binding domain of MYB68 and 68% of similarity and 75% of identity with MYB84 from *A. thaliana*. Since this is the first R2R3-MYB transcription factor isolated for *Q. suber* we named it *QsMYB1*. The upstream sequence obtained includes a putative promoter region of about 900 bp. *In silico* analysis of this region revealed the presence of consensus motifs and transcription factor binding sites (Fig. 2).

RACE results confirmed the presence of two transcripts (named *QsMYB1.1* and *QsMYB1.2)* differing only in sequence length of the 5'-UTR (289 bp for *QsMYB1.1* and 375 bp for *QsMYB1.2*). As the position of the start codon is not altered, the two transcripts are predicted to encode a similar protein. Sequence analysis showed that in transcript *QsMYB1.2* the intron I0 is retained whereas in transcript *QsMYB1.1* this intron is completely spliced. Moreover, the I0 intron contains a SSR, namely a dinucleotide $(CT)_n$ repeat.

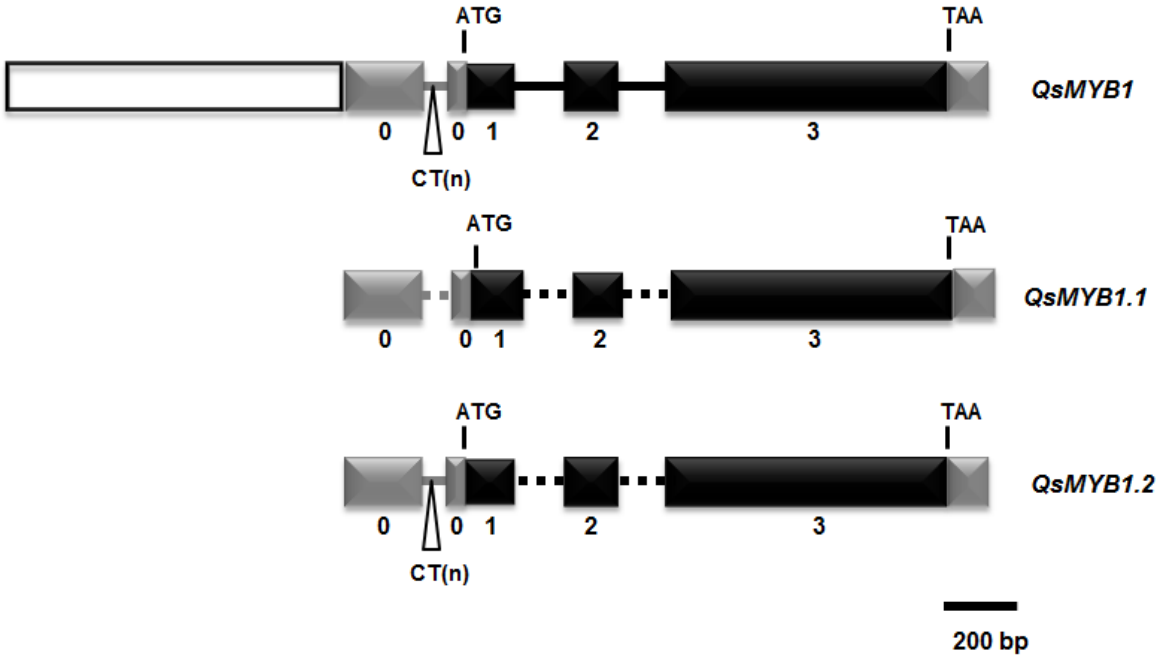


**Fig. 1**. Schematic representation of the *QsMYB1* genomic structure and the two alternatively spliced transcripts (*QsMYB1.1* and *QsMYB1.2*). Exons are numbered and represented by filled boxes; introns are shown as horizontal lines. The spliced introns are represented as dashed lines. The untranslated regions (5' and 3'-UTRs) are in dark grey and the coding region in black. The start (ATG) and the stop codon (TAA) are indicated. The putative promoter region is represented by an open box in the upstream region.

### 3.2. R2R3-MYB domain identification

Based on published data (Stracke et al. 2001) the R2R3 domain was identified within the N-terminus of QsMYB1 protein sequence. The R2 repeat is comprised between the amino acids 12 and 65 and R3 goes from the amino acids 66 to 116. MYB68 protein from *Arabidopsis* together with MYB84, MYB87, MYB36, MYB37 and MYB38 proteins all fall within subgroup-14 (Krantz et al. 1998; Stracke et al. 2001). The alignment of QsMYB1 protein sequence with members of MYB subgroup-14 from *Arabidopsis* (Fig. S1A, supplemental data) revealed a high identity with the consensus sequence, [W]-x(20)-[W]-x(19)-[W]-x(12)-[F]-x(18)-[W]-x(18)-[W], corresponding to the DNA binding domain with the characteristic tryptophan residues. However, QsMYB1 lacked two shared motif at C-terminus, SFSQLLLDPN and TSTADQSTISWEDI, found in some proteins of this MYB subgroup in *Arabidopsis* (Kranz et al. 1998). Moreover, the alignment of QsMYB1 amino acid sequence with some R2R3-MYB transcription factors of other tree genera, namely, *Populus, Picea and Pinus* (Fig. S1B, supplemental data) also showed a high similarity within the R2R3 domain.


**Fig. 2**. Sequence of *QsMYB1* promoter. Some of the putative *cis*-elements identified by the PlantPan interface (Chang et al. 2008) are numbered and shown in boxes. **1)** Athb-1 (cell differentiation); **2)** ACGTATERD1 (stress response); **3)** RAV1 (hormone signaling); **4)** RAV1-A (hormone signaling); **5)** ATHB-5 (hormone signaling); **6)** RAV1-B (hormone signaling); **7)** MYB-PLANT (phenylpropanoids pathway; **8)** ATHB-9 (cell differentiation); **9)** ARR10 (response regulator); **10)** GATAbox (light response, tissue specific expression); **11)** CARGATconsensus (flowering time); **12)** ARR1AT (response regulator); **13)** GT1-consensus (light response); **14)** MYB-4 (stress response); **15)** MYCCONSENSUSAT (stress response); **16)** LTRECOREATCOR15 (stress response). A complete list of the *cis*-acting elements is provided as supplemental data (Table S1).

### 3.3. Expression study by RT-qPCR

Relative transcript abundance of *QsMYB1.1* and *QsMYB1.2* was evaluated in several organs and tissues from *Q. suber* by RT-qPCR with transcript specific primers. Regarding the group of tissues (Fig. 3A), results revealed that the two splicing variants were preferentially accumulated in the newly synthesized phellem harvested from trees producing the three types of cork, exhibiting an expression level several fold higher when compared to the other samples. As an example, *QsMYB1.1* transcript was, respectively, 869-fold and 1476-fold more abundant in virgin and reproduction cork than in wood. However, the expression of *QsMYB1* in phellem harvested from branches showed only differences of 176-fold (*QsMYB1.1*) and 119-fold (*QsMYB1.2*) compared to wood. It is noteworthy that whereas the expression of *QsMYB1.1* was significantly different among the three cork types, the unspliced variant (*QsMYB1.2*) had statistically identical abundances in VC when compared to SC and RC, whereas SC and RC showed different levels of expression between them. In addition, the results also showed that the *QsMYB1.1* transcript was more abundant than *QsMYB1.2*. In the group of organs (Fig. 3B), the expression detected in roots, was very low for both transcripts. In fact, for the unspliced transcript (*QsMYB1.2*) the Ct values obtained for this sample were higher than 35 and, due to the poor precision in this range, the fold change in expression of young-branches versus roots was not calculated. But, with respect to *QsMYB1.1* transcript, the results showed that the transcript abundance in young branches was 7-fold higher than in roots. Regarding the other organs, namely, leaves, male flowers and immature acorns neither of *MYB1* transcripts was found to be expressed.

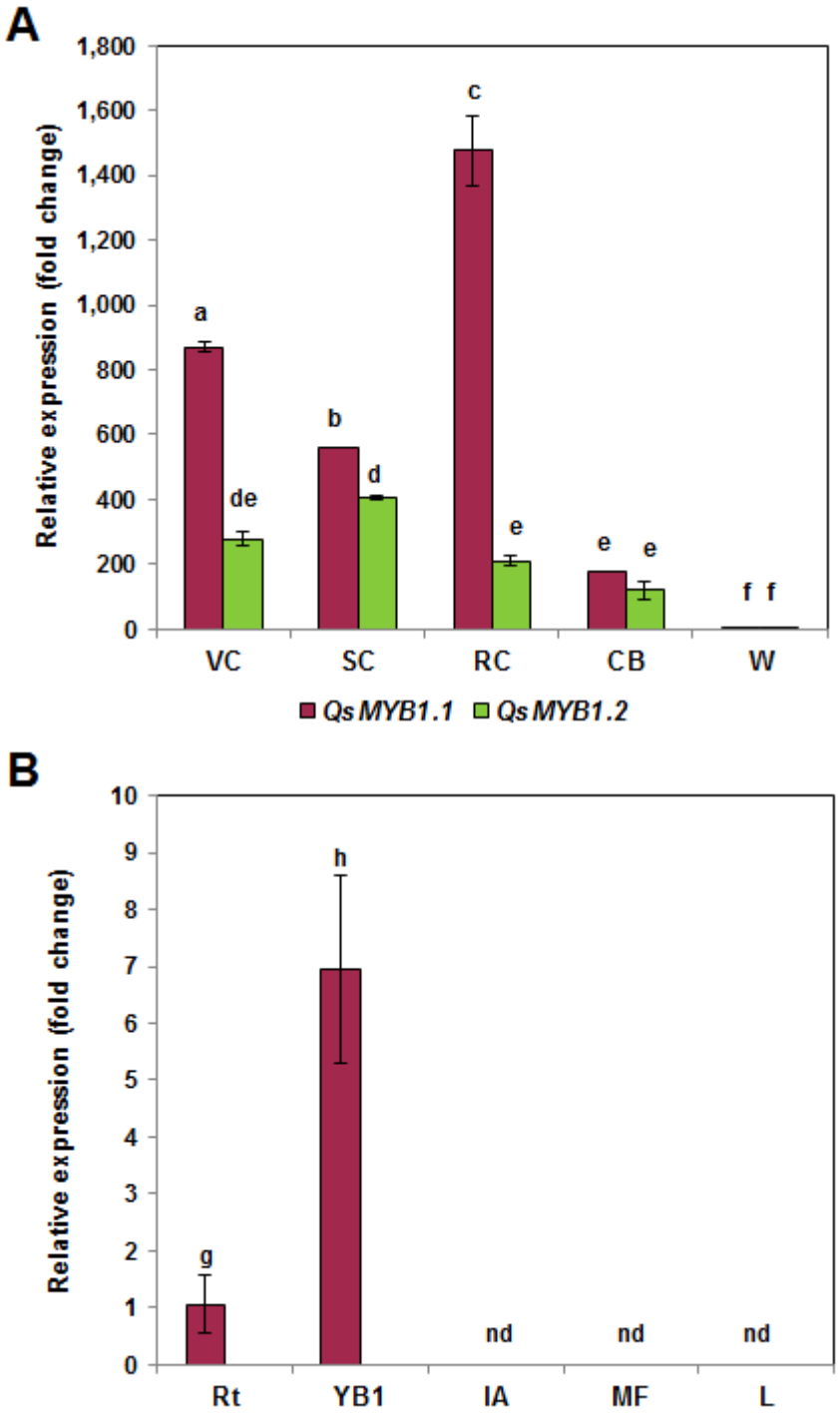


**Fig. 3**. Relative transcript abundance of *QsMYB1* transcripts in *Q. suber* **(A)** tissues and **(B)** organs assessed by RT-qPCR. The data represent the mean ± SD (n=3). The geometric mean of *ACT* and *EF-1α* genes was used to normalize data. Statistical significant differences between relative transcripts abundances were evaluated by a one-way Analysis of variance (ANOVA) (GraphPad Software, Inc) employing the Tukey's test. The level of statistical significance was set at $P < 0.05$. VC, virgin cork; SC, second cork; RC, reproduction cork; CB, cork branch; W, wood; Rt, roots; YB1, one-year-old young branch; IA, immature acorn; MF, male flower; L, leaves; nd, not detected.

## 4. Discussion

### 4.1. QsMYB1 is an R2R3-MYB transcription factor that possible undergoes alternative splicing

The results indicate that *QsMYB1* gene may be alternatively spliced into two different mRNA variants differing in length and sequence of the 5' UTR due to retention of an intron. These results suggest a regulatory mechanism associated with QsMYB1. In *Arabidopsis* and rice, the expression of AS variants has been demonstrated to be controlled in a developmental, tissue or subcellular-specific manner (Koo et al. 2007, Zhang et al. 2009). Moreover, several studies have shown that the presence of an intron within 5' and 3'-UTRs may have a regulatory function by influencing mRNA stability, transport and/or translation efficiency (Samader et al. 2008, Xue et al. 2008).

Another interesting feature observed, was the presence of a SSR within the retained intron. An increasing number of SSRs has been characterized in protein coding sequences and non-coding regions (promoters, UTRs) of genes in several plant species (Parida et al. 2009). A recent study has demonstrated that the length of $(AT)_n$ insertions inside the promoter region could modulate up or down the *Gmhsp17.6-L* gene in soybean (Fuganti et al. 2010). Chung et al. (2006) have also concluded that in the *Arabidopsis EF1$\alpha$-A3* gene, the presence of a 5'-UTR intron modulated its expression in a size dependent manner. Therefore, we hypothesize that the SSR in the leader intron may be acting as a *cis*-element in the regulation of *QsMYB1* expression. Interestingly, the expression data showed that the *QsMYB1.1* transcript was the one with the highest level of abundance in all tissues and organs with expression. As mentioned above, this expression pattern of both splicing variants may be altered by particular conditions (e.g. stress, developmental stage) as already described by some authors (Egawa et al. 2006, Lin et al. 2009). However, further studies are needed to fully understand the role of the intron as well as the AS mechanism during the transcriptional or posttranscriptional process of *QsMYB1*.

### 4.2. QsMYB1 is mainly expressed in cork tissues

The expression profile of *QsMYB1* indicates that this transcription factor is mainly expressed in organs and tissues with secondary growth resulting from the activity of the phellogen. Comparing the relative transcript abundance in the highly suberized tissue, cork, with the lignified and non-suberin producing tissue, wood, we found that *QsMYB1* was much more expressed in cork than wood which is consistent with the results reported by Soler et al. (2007). In a preceding study in *Arabidopsis* (Feng et al. 2004), the authors found that the putative orthologous of QsMYB1, the *AtMYB68* is specifically expressed in root pericycle cells, at the side of the secondary root formation and surprisingly not in the endodermis. Moreover, the authors have compared lignin levels of root cultures from *myb68* mutant versus wild type and interestingly found that, the mutant

produced an increased lignin level and thus suggested that the *AtMYB68* may function directly or indirectly to repress some aspect of lignin production.

Of note are also the results obtained for cork collected from branches, namely, the differences in expression level of *QsMYB1.1* compared to the ones detected in other cork samples (VC, SC and RC). On the other hand, in *QsMYB1.2* those differences were statistically significant only in comparison with SC. Though there is not a clear trend, one possible reason for these differences may be the developmental stage of cork from stem versus cork from the branch. Phellogen forms in cork oak plants during the first year of growth and at this stage some phellem cells are already observed. However, it is during the $5^{th}$ -$7^{th}$ years that phellem cells acquire the characteristics typical of "adult" cork cells and phellogen produces a higher number of these cells (Graça and Pereira 2004). The differential expression of each AS variant in VC, SC and RC may be also related to the differences in the chemical composition of each cork type that have already been reported (Pereira 1988). Regarding the results obtained for the organs group, the higher expression level of *QsMYB1.1* observed in young branches compared to roots may also be related to the number of suberized cells present in both organs, since it is expected that branches (one year-old) produce more suberized cells than the root with 15 days. The *in silico* analysis of *QsMYB1* promoter region showed the presence of several putative *cis*-acting regulatory elements related to phenylpropanoid pathway, which includes some of the basics reactions of the poly(phenolic) domain synthesis from suberin and lignin. This reinforces the assumption that QsMYB1 may be regulating one or more metabolic pathways involved in cork formation. However other studies, namely, functional studies are required to determine its exact function.

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## Supplemental data

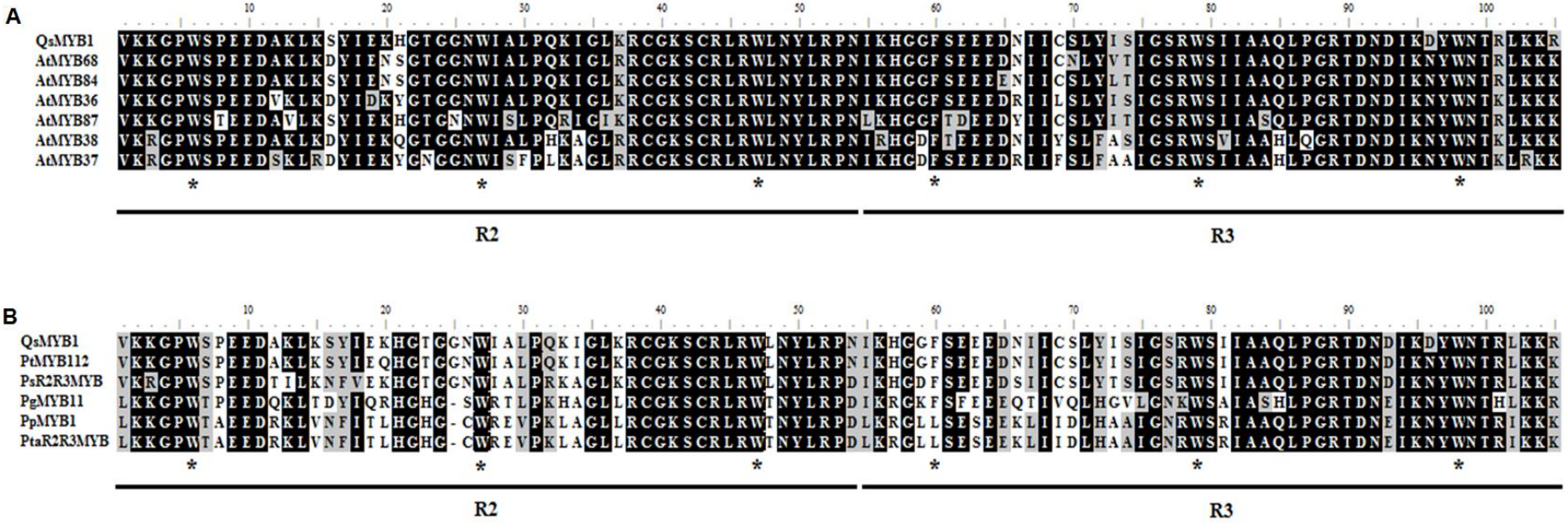


**Fig. S1**. **(A)** Comparison of amino acids sequences of the R2 and R3 repeats from QsMYB1 with those of MYB subgroup - 14 members from *Arabidopsis*. Black and grey shadings represent identical and similar amino acids, respectively (threshold shading set as 70%). The characteristic ([W]-x(20)-[W]-x(19)-[W]-x(12)-[F]-x(18)-[W]-x(18)-[W]) consensus motif of MYB subgroup-14 are marked with an asterisk (*). The MYB proteins from *A. thaliana* were the following: AtMYB68 (NP_201380), AtMYB87 (NP_195492), AtMYB38 (1009116707), AtMYB37 (NP_197691), AtMYB36 (200570), AtMYB84 (NP_ 190538). **(B)** Alignment of amino acids sequences of the R2 and R3 repeats from QsMYB1 with the R2R3-MYB domain of R2R3-MYB transcription factors from other trees genera. Black and grey shadings represent identical and similar amino acids, respectively (threshold shading set as 70%). The compared R2R3-MYB TFs were: *Populus trichocarpa* MYB112 (PtMYB112, XP_002301137.1), *Picea sitchensis* R2R3MYB (PsR2R3MYB, ABR16159.1), *Picea glacua* MYB11 (PgMYB11, ABQ51227.1), *Pinus pinaster* MYB1 (PpMYB1, ACA33839.1), *Pinus taeda* R2R3MYB (PtaR2R3MYB, AAQ62541.1).

**Table A1.** Results of *QsMYB1* promoter analysis using the PlantPan interface (http://plantpan.mbc.nctu.edu.tw/) to identify the putative *cis*-elements. The *cis*-elements shown in Fig. 2 are indicated in bold.

| ***Cis*-element** | **Site** | **Strand** | **Sequence** | **Species** |
|---|---|---|---|---|
| AGL3 | 692 | - | aatatacaaTATGGgatt | Arabidopsis |
| **Athb-1 (1)** | 32 | + | **tttcaATTATtaga** | Arabidopsis |
| Athb-1 | 142 | + | tttaaATTATttta | Arabidopsis |
| Athb-1 | 217 | + | aatatATTATttct | Arabidopsis |
| Athb-1 | 285 | - | attgATAATtggtg | Arabidopsis |
| Athb-1 | 429 | - | tacgATAATtagtt | Arabidopsis |
| Athb-1 | 446 | - | accaATAATggagt | Arabidopsis |
| Athb-1 | 637 | + | attaaATTATtttt | Arabidopsis |
| Athb-1 | 686 | - | atttATAATataca | Arabidopsis |
| Athb-1 | 769 | + | catcgATTATattt | Arabidopsis |
| Athb-1 | 824 | + | tataaATTATtttt | Arabidopsis |
| ATHB-5 | 3 | + | ttaTTATTg | Arabidopsis |
| ATHB-5 | 35 | + | caaTTATTa | Arabidopsis |
| **ATHB-5 (5)** | 145 | + | **aaaTTATTt** | Arabidopsis |
| ATHB-5 | 150 | + | attTTATTg | Arabidopsis |
| ATHB-5 | 195 | + | gatTTATTg | Arabidopsis |
| ATHB-5 | 220 | + | ataTTATTt | Arabidopsis |
| ATHB-5 | 252 | + | actTTATTt | Arabidopsis |
| ATHB-5 | 263 | - | cAATAAagt | Arabidopsis |
| ATHB-5 | 391 | - | tAATAAatt | Arabidopsis |
| ATHB-5 | 448 | - | cAATAAtgg | Arabidopsis |
| ATHB-5 | 474 | + | cacTTATTc | Arabidopsis |
| ATHB-5 | 589 | - | tAATAAatt | Arabidopsis |
| ATHB-5 | 632 | + | ctcTTATTa | Arabidopsis |
| ATHB-5 | 640 | + | aaaTTATTt | Arabidopsis |
| ATHB-5 | 647 | + | tttTTATTt | Arabidopsis |
| ATHB-5 | 651 | + | tatTTATTt | Arabidopsis |
| ATHB-5 | 655 | + | tatTTATTt | Arabidopsis |
| ATHB-5 | 681 | + | tttTTATTt | Arabidopsis |
| ATHB-5 | 787 | + | cctTTATTa | Arabidopsis |
| ATHB-5 | 827 | + | aaaTTATTt | Arabidopsis |
| ATHB-9 | 169 | + | acaactaATGATaacctac | Arabidopsis |
| **ATHB-9 (8)** | 229 | + | **ctcatatATGATaaagata** | Arabidopsis |
| ATHB-9 | 498 | + | acaaacaATGATattgaac | Arabidopsis |
| ATHB-9 | 564 | + | tcaattgATGATatataag | Arabidopsis |
| ATHB-9 | 711 | - | aatttttATCATcatatca | Arabidopsis |

**Table A1.** (cont.)

| ***Cis*-element** | **Site** | **Strand** | **Sequence** | **Species** |
|---|---|---|---|---|
| ATHB-9 | 714 | - | ttttatcATCATatcaaga | Arabidopsis |
| ATHB-9 | 760 | - | tttttatATCATcgattat | Arabidopsis |
| **RAV1 (3)** | 67 | + | **tttCAACAacat** | Arabidopsis |
| RAV1 | 70 | + | caaCAACAtatt | Arabidopsis |
| **ACGTATERD1 (2)** | 54 | + | **ACGT** | Arabidopsis |
| ACGTATERD1 | 54 | - | ACGT | Arabidopsis |
| ANAERO1CONSENSUS | 875 | + | AAACAAA | maize/Arabidopsis/pea/barley/rice |
| ARR10 | 243 | + | AGATATTT | Arabidopsis |
| **ARR10 (9)** | 305 | + | **AGATTCGA** | Arabidopsis |
| ARR10 | 730 | + | AGATATTA | Arabidopsis |
| ARR1AT | 112 | + | TGATT | Arabidopsis |
| ARR1AT | 194 | + | AGATT | Arabidopsis |
| ARR1AT | 305 | + | AGATT | Arabidopsis |
| **ARR1AT (12)** | 465 | + | **GGATT** | Arabidopsis |
| ARR1AT | 562 | - | AATCA | Arabidopsis |
| ARR1AT | 705 | + | GGATT | Arabidopsis |
| ARR1AT | 772 | + | CGATT | Arabidopsis |
| ARR1AT | 887 | + | AGATT | Arabidopsis |
| ASF1MOTIFCAMV | 52 | + | TGACG | tobacco/Arabidopsis |
| ATHB6COREAT | 35 | + | CAATTATTA | Arabidopsis |
| ATHB6 | 35 | + | CAATTATTA | Arabidopsis |
| Agamous | 446 | - | ACCAATAATGG | Arabidopsis |
| Agamous | 447 | + | CCAATAATGGA | Arabidopsis |
| Agamous | 787 | + | CCTTTATTAGA | Arabidopsis |
| Athb-1 | 35 | + | CAATTATT | Arabidopsis |
| Bellringer | 137 | - | TCTAATTT | Arabidopsis |
| Bellringer | 593 | + | AAATTAAT | Arabidopsis |
| Bellringer | 670 | + | AAATTAAT | Arabidopsis |
| Bellringer | 708 | - | TTTAATTT | Arabidopsis |
| Bellringer | 79 | - | ATTAATTT | Arabidopsis |
| C8GCARGAT | 35 | + | CAATTATTAG | Arabidopsis |
| C8GCARGAT | 35 | - | CAATTATTAG | Arabidopsis |
| **CARGATCONSENSUS (11)** | 447 | + | **CCAATAATGG** | Arabidopsis |
| CARGATCONSENSUS | 447 | - | CCAATAATGG | Arabidopsis |
| CARGCW8GAT | 35 | + | CAATTATTAG | Arabidopsis |
| CARGCW8GAT | 35 | - | CAATTATTAG | Arabidopsis |
| Core | 139 | - | TAAT | Arabidopsis |
| Core | 147 | + | ATTA | Arabidopsis |

**Table A1.** (cont.)

| ***Cis*-element** | **Site** | **Strand** | **Sequence** | **Species** |
|---|---|---|---|---|
| Core | 174 | - | TAAT | Arabidopsis |
| Core | 222 | + | ATTA | Arabidopsis |
| Core | 290 | - | TAAT | Arabidopsis |
| Core | 340 | + | ATTA | Arabidopsis |
| Core | 37 | + | ATTA | Arabidopsis |
| Core | 391 | - | TAAT | Arabidopsis |
| Core | 397 | + | ATTA | Arabidopsis |
| Core | 40 | + | ATTA | Arabidopsis |
| Core | 434 | - | TAAT | Arabidopsis |
| Core | 436 | + | ATTA | Arabidopsis |
| Core | 442 | - | TAAT | Arabidopsis |
| Core | 451 | - | TAAT | Arabidopsis |
| Core | 490 | - | TAAT | Arabidopsis |
| Core | 5 | + | ATTA | Arabidopsis |
| Core | 589 | - | TAAT | Arabidopsis |
| Core | 595 | + | ATTA | Arabidopsis |
| Core | 597 | - | TAAT | Arabidopsis |
| Core | 637 | + | ATTA | Arabidopsis |
| Core | 642 | + | ATTA | Arabidopsis |
| Core | 672 | + | ATTA | Arabidopsis |
| Core | 674 | - | TAAT | Arabidopsis |
| Core | 691 | - | TAAT | Arabidopsis |
| Core | 710 | - | TAAT | Arabidopsis |
| Core | 734 | + | ATTA | Arabidopsis |
| Core | 736 | - | TAAT | Arabidopsis |
| Core | 774 | + | ATTA | Arabidopsis |
| Core | 79 | + | ATTA | Arabidopsis |
| Core | 792 | + | ATTA | Arabidopsis |
| Core | 81 | - | TAAT | Arabidopsis |
| Core | 829 | + | ATTA | Arabidopsis |
| Core | 889 | + | ATTA | Arabidopsis |
| EVENINGAT | 243 | - | AGATATTTT | Arabidopsis/eggplant |
| GAREAT | 891 | + | TAACAAA | Arabidopsis |
| **GATABOX (10)** | 178 | + | **GATA** | petunia/Arabidopsis/rice |
| GATABOX | 238 | + | GATA | petunia/Arabidopsis/rice |
| GATABOX | 244 | + | GATA | petunia/Arabidopsis/rice |
| GATABOX | 288 | + | GATA | petunia/Arabidopsis/rice |
| GATABOX | 432 | + | GATA | petunia/Arabidopsis/rice |
| GATABOX | 507 | + | GATA | petunia/Arabidopsis/rice |

**Table A1.** (cont.)

| ***Cis*-element** | **Site** | **Strand** | **Sequence** | **Species** |
|---|---|---|---|---|
| GATABOX | 573 | + | GATA | petunia/Arabidopsis/rice |
| GATABOX | 717 | - | TATC | petunia/Arabidopsis/rice |
| GATABOX | 725 | - | TATC | petunia/Arabidopsis/rice |
| GATABOX | 731 | + | GATA | petunia/Arabidopsis/rice |
| GATABOX | 766 | - | TATC | petunia/Arabidopsis/rice |
| GBF5 | 738 | - | ATGAGT | Arabidopsis |
| GT1CONSENSUS | 205 | + | GAAAAT | pea/oat/rice/tobacco/Arabidopsis |
| GT1CONSENSUS | 238 | + | GATAAA | pea/oat/rice/tobacco/Arabidopsis |
| GT1CONSENSUS | 288 | + | GATAAT | pea/oat/rice/tobacco/Arabidopsis |
| GT1CONSENSUS | 322 | - | ATTTCC | pea/oat/rice/tobacco/Arabidopsis |
| GT1CONSENSUS | 432 | + | GATAAT | pea/oat/rice/tobacco/Arabidopsis |
| **GT1CONSENSUS (13)** | 488 | + | **GGTAAT** | pea/oat/rice/tobacco/Arabidopsis |
| GT1CONSENSUS | 554 | + | GAAAAA | pea/oat/rice/tobacco/Arabidopsis |
| GT1CONSENSUS | 715 | - | TTTATC | pea/oat/rice/tobacco/Arabidopsis |
| GT1CONSENSUS | 753 | - | TTTTTC | pea/oat/rice/tobacco/Arabidopsis |
| HEXAMERATH4 | 814 | - | CGACGG | Arabidopsis |
| **LTRECOREATCOR15 (16)** | 813 | + | **CCGAC** | Arabidopsis/rape |
| MYB1LEPR | 545 | + | GTTAGTT | Arabidopsis/tomato |
| MYB2CONSENSUSAT | 326 | - | CCGTTG | Arabidopsis |
| MYB4 | 182 | + | ACCTACC | Arabidopsis |
| MYB4 | 402 | - | GTTAGGT | Arabidopsis |
| **MYB4 (14)** | 497 | + | **AACAAAC** | Arabidopsis |
| MYB4 | 545 | - | GTTAGTT | Arabidopsis |
| MYBCOREATCYCB1 | 326 | - | CCGTT | Arabidopsis |
| MYBCORE | 326 | + | CCGTTG | Arabidopsis/petunia |
| **MYBPLANT (7)** | 181 | + | **AACCTACC** | snapdragon/bean/petunia/ Arabidopsis |
| MYBPLANT | 402 | - | GTTAGGTT | snapdragon/bean/petunia/ Arabidopsis |
| MYCCONSENSUSAT | 161 | + | CACCTG | Arabidopsis |
| MYCCONSENSUSAT | 161 | - | CACCTG | Arabidopsis |
| **MYCCONSENSUSAT (15)** | 565 | + | **CAATTG** | Arabidopsis |
| MYCCONSENSUSAT | 565 | - | CAATTG | Arabidopsis |
| POLASIG1 | 152 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 197 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 2 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 254 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 264 | + | AATAAA | pea/rice/Arabidopsis |

**Table A1.** (cont.)

| ***Cis*-element** | **Site** | **Strand** | **Sequence** | **Species** |
|---|---|---|---|---|
| POLASIG1 | 392 | + | AATAAA | pea/rice/Arabidopsis |
| POLASIG1 | 590 | + | AATAAA | pea/rice/Arabidopsis |
| POLASIG1 | 649 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 653 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 657 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 683 | - | TTTATT | pea/rice/Arabidopsis |
| POLASIG1 | 789 | - | TTTATT | pea/rice/Arabidopsis |
| PREATPRODH | 738 | - | ATGAGT | Arabidopsis |
| **RAV1-A (4)** | 70 | + | **CAACA** | Arabidopsis |
| RAV1-A | 73 | + | CAACA | Arabidopsis |
| **RAV1-B (6)** | 161 | + | **CACCTG** | Arabidopsis |
| RAV1AAT | 70 | + | CAACA | Arabidopsis |
| RAV1AAT | 73 | + | CAACA | Arabidopsis |
| RAV1BAT | 161 | + | CACCTG | Arabidopsis |
| TAAAGSTKST1 | 1 | - | CTTTA | potato |
| TAAAGSTKST1 | 240 | + | TAAAG | potato |
| TAAAGSTKST1 | 253 | - | CTTTA | potato |
| TAAAGSTKST1 | 266 | + | TAAAG | potato |
| TAAAGSTKST1 | 538 | + | TAAAG | potato |
| TAAAGSTKST1 | 788 | - | CTTTA | potato |
| TBOXATGAPB | 371 | - | CAAAGT | Arabidopsis |
| TBOXATGAPB | 533 | - | CAAAGT | Arabidopsis |
| TBOXATGAPB | 878 | - | CAAAGT | Arabidopsis |
| WBOXATNPR1 | 51 | + | TTGAC | Arabidopsis |

**(1)** Cell differentiation, **(2)** stress response, **(3)** hormone signaling, **(4)** hormone signaling, **(5)** hormone signaling, **(6)** hormone signaling, **(7)** phenylpropanoid pathway, **(8)** cell differentiation, **(9)** response regulator, **(10)** light response, **(11)** flowering time, **(12)** response regulator, **(13)** light response, **(14)** stress response, **(15)** stress response, **(16)** stress response.

# CHAPTER III

## *QsMYB1* expression is modulated in response to heat and drought stresses and during plant recovery in *Quercus suber*

*Chapter published as an original article in a SCI journal:*

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## Abstract

Cork oak is an economically important forest species showing a great tolerance to high temperatures and shortage of water. However, the mechanisms underlying this plasticity are still poorly understood. Among the stress regulators, transcription factors (TFs) are especially important since they can control a wide range of stress-inducible genes, which make them powerful targets for genetic engineering of stress tolerance. Here we evaluated the influence of increasing temperatures (up to 55 ºC) or drought (18 % field capacity, FC) on the expression profile of an R2R3-MYB transcription factor of cork oak, the QsMYB1. *QsMYB1* was previously identified as being preferentially expressed in cork tissues and as having an associated alternative splicing mechanism, which results in two different transcripts (*QsMYB1.1* and *QsMYB1.2*). Expression analysis by reverse transcription quantitative PCR (RT-qPCR) revealed that increasing temperatures led to a gradual down-regulation of *QsMYB1* transcripts with more effect on *QsMYB1.1* abundance. On the other hand, under drought condition, expression of *QsMYB1* variants, mainly the *QsMYB1.2*, was transiently up-regulated shortly after the stress imposition. Recovery from each stress has also resulted in a differential response by both *QsMYB1* transcripts. Several physiological and biochemical parameters (plant water status, chlorophyll fluorescence, lipid peroxidation and proline content) were determined in order to monitor the plant performance under stress and recovery. In conclusion, this report provides the first evidence that QsMYB1 TF may have a putative function in the regulatory network of cork oak response to heat and drought stresses and during plant recovery.

***Keywords:*** cork oak, R2R3-MYB, abiotic stress, recovery, gene expression

***Abbreviations*****:** DW, dry weight; F, steady state fluorescence; F0, minimum fluorescence; FC, field capacity; 5'UTR, 5'-untranslated region; Fm, maximum fluorescence; Fv, variable fluorescence; Fv/Fm, maximum quantum yield of PSII photochemistry; FW, tissue fresh weight; gDNA, genomic DNA; MDA, malondialdehyde; ΦPSII , quantum yield of PSII photochemistry; RT-qPCR, reverse transcription quantitative PCR; RWC, relative water content; TFs, transcription factors; TW, turgid weight; WS, water stress; WW, well watered.

## 1. Introduction

Climate changes are expected to severely affect Southern Europe where increasing high temperatures and shortage of water will have an impact on plant production (IPCC 2007). To cope with adverse environmental conditions plants have evolved a wide spectrum of molecular mechanisms to sense, change rapidly and adapt accordingly (Ahuja et al. 2010). In plants under abiotic stresses, various biochemical and physiological responses are induced (Oono et al. 2003), but one crucial element of all stress responses is the ability to modify the transcriptome (Walley and Dehesh 2010). In spite of its importance, many of these regulatory mechanisms are still unknown or little understood, especially in forest trees. Thus, new insights into the adaptive strategies applied by the plants in response to abiotic stresses could be a step forward to improve plant tolerance in a climate change scenario.

Cork oak (*Quercus suber* L.) forest is widely distributed in the coastal regions of the western Mediterranean basin (Gil and Varela 2008) and is considered one of the few examples of fully sustainable forestry exploitation, mainly due to the production of cork (Pereira 2007). Giving its geographic distribution, *Q. suber* evolved and became tolerant to hot and dry summers and to soils of low fertility, showing even a xerophytic character (Molinas and Verdaguer 1993a;b, Pereira 2007). Cork results from the activity of a secondary meristem, the cork cambium (or phellogen) that produces a layer of cork each year (Pereira 2007). It is known that the cork cambium dynamics are sensitive to climatic factors, namely: the precipitation, which exerts a large and positive influence on cork growth; drought, which is a limiting factor; and extreme temperatures that generally have a negative effect on cork growth (Caritat et al. 2000). The knowledge on fundamental processes related to the biosynthesis and differentiation of cork must therefore be increased in order to develop tools and technologies to support future breeding programs in cork oak, where the regulatory mechanisms of response to abiotic stresses (e.g. drought, extreme temperatures or soil salinity) should be highlighted. A previous study performed by Chaves et al. (2011) concluded that contrasting temperatures (10 ºC and 28 ºC) have influence on *Q. suber* leaf metabolites (e.g. sucrose, quercitol and quinic acid) and also on expression of genes encoding key enzymes of secondary metabolism (e.g. Chorismate synthase, phenylalanine ammonia-lyase, NADPH-dependent cinnamyl alcohol dehydrogenase). Kwak et al. (2011) found that different intensities of drought and light have effect on physiological and biochemical aspects of cork oak seedlings. More recently, Correia et al. (2013) have verified that cork oak leaves experience interrelated and specific DNA methylation and histone H3 acetylation changes due to elevated temperature conditions.

Plants have developed regulatory circuits, which include stress sensors, signaling pathways comprising a network of protein-protein interactions, TFs and promoters, and finally the output proteins or metabolites (Ciarmiello et al. 2013). Because TFs, contrarily to most structural genes, tend to control multiple pathways, they have emerged as powerful tools for the manipulation of complex metabolic pathways in plants (Hussain et al. 2011). In plant response to abiotic stresses,

at least four different regulons can be identified: the CBF/DREB regulon; the NAC (NAM, ATAF and CUC) and ZF-HD (zinc-finger homeodomain) regulon; the AREB/ABF (ABA responsive element-binding protein / ABA- binding factor) regulon; and the MYC (myelocytomatosis oncogene) / MYB (myeloblastosis oncogene) regulon (Saibo et al 2009). Previously, we reported the molecular characterization of an R2R3-MYB transcription factor, the QsMYB1 (Almeida et al. 2013). The results showed that *QsMYB1* is up-regulated in cork tissues, suggesting its putative involvement in cork biosynthesis process. Moreover, a possible splicing mechanism is also associated with its regulatory function and two different transcripts were identified (*QsMYB1.1* and *QsMYB1.2*) differing only in the 5'-untranslated region (5'UTR). In transcript *QsMYB1.1* the 5'UTR intron is spliced while in the *QsMYB1.2* this intron is retained. Additionally, the in silico analysis of the *QsMYB1* putative promoter region revealed that several *cis*-acting elements related to stress and hormones responses were present. In fact, several members of the R2R3-MYB family have already been implicated in stress responses. Examples of these TFs include: the AtMYB96 whose over-expressing mutant showed enhanced drought resistance (Seo et al. 2009); the AtMYB60 which demonstrated to be negatively modulated by drought (Cominelli et al. 2005); the AtMYB68 whose loss function mutant showed a reduce ability to deal with high temperatures (Feng et al. 2004); and some members of MYB subgroup-4 from *Picea glauca* and *Pinus taeda* that showed to have stress responsive transcript profiles after wounding or exposure to cold (Bedon et al. 2010).

Based on our previous findings and on the fact that, during cork biosynthesis period, cork oak is commonly exposed to high temperatures and drought this work aimed to evaluate if QsMYB1 could be considered as a putative player in the regulatory network of cork oak response to these abiotic stresses and in the recovery process. Expression analysis by RT-qPCR showed that both stress stimuli differentially modulated the expression pattern of the two *QsMYB1* splicing variants. The results also showed that this pattern is changed in both transcripts during recovery.

## 2. Materials and methods

### 2.1. Plant material and stress assay experimental design

#### 2.1.1. Drought stress

Seed-grown twelve-month-old cork oak plants were acquired from a forest plant producer (Portugal). A month before the drought stress experiment, plants were potted in 1L containers with the same weight of a peat/perlite mixture (1/1) and transferred from semi controlled greenhouse conditions to a climate chamber for acclimation. During the acclimation period, plants were fertilized twice and growth conditions were adjusted to 25 / 20 ºC (day / night), 60-70 % relative humidity, 16 h light photoperiod and 250 µmol $m^{-2}$ $s^{-1}$ of photosynthetic photon flux. Previously to drought treatment, all plants were watered to 60 % FC and the stress was initiated by withholding

water until soil moisture reach 18 % FC (water stress group, WS) which took five days (Supplemental data, Fig. S1A). A well watered group of plants (control group, WW) was held at 60% FC during the entire assay. The percentages of FC were maintained by adding the amount of water lost by evapotranspiration that was monitored by weighing the containers every day. The drought stress was imposed for 30 days and after that, stressed plants were re-watered until well-watered conditions. For analysis, two pools (6 plants each) of stems and leaves were collected from WW and WS plants at four points: (T1) the first day of WS conditions; (T2) 30 days after T1; (R1) one day after re-watering; and (R2) one week after re-watering. After collection, stem and leaf samples were immediately frozen in liquid nitrogen and kept at -80 ºC until further use. At the same experimental points, fresh leaves (fully expanded) were used for water status and fluorescence related parameters.

### 2.1.2. Cumulative heat stress

Seed grown ten-month-old cork oak plants were acquired from a forest plant producer (Portugal) and transferred from semi controlled greenhouse conditions to a climate chamber for a 2 weeks acclimation period. During this period, the climate chamber environment was kept constant at 25 ºC / 20 ºC (day / night) of air temperature, 60-70 % relative humidity, 250 µmol $m^{-2}$ $s^{-1}$ of photosynthetic photon flux, 16 h light photoperiod. Plants were daily watered in order to maintain 60 % FC. During the experimental treatment, relative humidity, irradiance, watering and photoperiod were held constant, while air temperature was gradually increased by 10 ºC every 3 days from 25 ºC to 55 ºC (Supplemental data, Fig. S1B). The minimum air temperature was 20 ºC at night (8 h). At each point (25 ºC, 35 ºC, 45 ºC and 55 ºC), peak temperature was lasted for 3 h before starting the next temperature ramp. Sampling was performed on the third day, during the peak heat hours (around 12 a.m.). At the end of the stress period, plants were allowed to recover at 25 ºC during one month (recovery-stress group, R55 ºC). Another group of plants were maintained at 25 ºC / 20 ºC (day / night) during the entire experience as control (recovery-control group, R25 ºC). At each point of analysis, three pools of stems (10 plants each) and leaves (6 plants each) were collected, immediately frozen in liquid nitrogen and kept at -80 ºC until subsequent analysis. At the same experimental points, fresh leaves (fully expanded) were used for water status and fluorescence related parameters.

### 2.2. Total RNA extraction and cDNA synthesis

Total RNA was extracted from stem samples using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) following manufacturer's instructions with minor modifications: addition of 50 µL from Plant RNA Aid solution (Ambion) to the lysis buffer and the use of 64 % (v/v) ethanol instead of binding solution. The protocol included also a step to remove genomic DNA (gDNA) using the On-column Dnase I digestion set (Sigma-Aldrich). A single RNA extraction was performed for each pool. At the

end, RNA samples corresponding to the same point of analysis were pooled together through a precipitation step with 10 M LiCl followed by washing with 70 % (v/v) ethanol and dissolution in Rnase free water. RNA concentration and purity were assessed with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) and the integrity was verified on a 1% (w/v) agarose gel. For cDNA synthesis only RNA samples with ratios of $A_{260/230}$ in the range of 2 - 2.2 and $A_{260/280}$ in the range of 1.8 - 2.2 were used. First-strand cDNAs were synthesized from 1.5 µg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen), which includes an additional gDNA elimination step and the use of a mix of oligo(dT) and random hexamer primers.

### 2.3. Analysis of QsMYB1 transcripts abundance by RT-qPCR

*QsMYB1* transcripts were specifically amplified using the primer QsMYB1fwd together with one of the following reverse oligonucleotides (Table 1): BNDrev to amplify the *QsMYB1.1* transcript (GenBank accession number JF970262) or the SSRrev for the amplification of *QsMYB1.2* transcript (GenBank accession number JF970263). The PCR mixture included 1X iQ™SYBR® Green Supermix (Bio-Rad Laboratories), 0.2 µM of each primer and 2 µL of the previously synthesized cDNA in a final volume of 20 µL. No template controls were also prepared by adding water instead of cDNA. Two independent analyses were performed with three technical replicates each. Absence of gDNA contamination was confirmed by RT-qPCR using a pair of primers (Ex1fwd / Ex2rev) spanning an intron in the coding region. The following PCR profile was used: an initial incubation at 95°C for 3 min, followed by 40 cycles with 10 s at 95 ºC, 15 s at 57 °C / 60 ºC (SSRrev / BNDrev) and 10 s at 72 °C with a single fluorescent reading taken at the end of each cycle. To distinguish specific from nonspecific products and primer dimers, a melting curve was obtained immediately after amplification. PCR and melting products were detected in real time with an iCycler iQ5 Instrument (Bio-Rad Laboratories). The threshold cycles ($C_T$) were calculated by the optical interface iQ5 software (Bio-Rad Laboratories). For each stress treatment four reference genes previously described (Soler et al. 2008, Marum et al. 2012) were tested (Table 2): β-tubulin (β-*TUB*), actin (*ACT*), polymerase elongation factor-1α (*EF*-1α) and clathrin adaptor complexes medium subunit family protein (*CACs*). PCR conditions and cycling parameters were the same as before with an annealing temperature of 60 °C. The same batch of cDNA was used on the amplification of target and reference genes. According to Normfinder analysis (Andersen et al. 2004), *CACs* gene was chosen as the most stable in drought stress conditions while in heat stress, β-*TUB* showed to be more stable. PCR runs from reference and target genes were done independently, using the same batch of cDNA. Amplification efficiencies (E) of primers were determined using the standard curve method with a 10-fold dilution series and calculated according to the equation $\% E = (10^{-1/slope}-1) \times 100$. All primers showed E values close to 100 %. Relative abundances of *QsMYB1* transcripts were calculated by the comparative $2^{-\Delta\Delta Ct}$ method (Livak and

Schmittgen 2001). Expression levels are represented as fold change relative to the respective control group in each sampling point.

**Table 1.** List of primers used in RT-qPCR to amplify *QsMYB1* transcripts and to detect gDNA contamination on cDNA samples.

| Transcript | Primer | Nucleotide sequence (5'-3') | Length of amplified fragment (bp) |
|---|---|---|---|
| ***QsMYB1.1*** | QsMyb1fwd | CCTTGCTCTCCTATTCTGGC | 118 |
| | BNDrev | TAGCTTCTCCCTATGGTTATTAGG | |
| ***QsMYB1.2*** | SSRrev | CCTCTGAAACCCAAATAAGAATAT | 195 |
| ***QsMYB1*** | Ex1fwd | GGGAGAGCTCCATGTTGTGAC | 174 - cDNA |
| | Ex2rev | CAAGAGTTGCCGCCTTAGATGGTTAA | 326 - gDNA |

**Table 2.** Description of *Q. suber* reference genes tested to normalize RT-qPCR results and the PCR amplification efficiency (E) of each primer pair.

| Primer | Gene | Nucleotide sequence (forward / reverse) | E (%) | Reference |
|---|---|---|---|---|
| β-TUB | β-tubulin | AAGAACATGATGTGCGCTGCT / TCCACCTCCTTGGTGCTCA | 97 | (Soler et al. 2008) |
| EF-1α | Elongation factor-1alpha | TTGTGCCGTCCTCATTATTGACT / TCACGGGTCTGACCATCCTT | 96 | (Soler et al. 2008) |
| ACT | Actin | TGACAATGGAACTGGAATGG / CATCACCAACATAGGCATCC | 94 | (Soler et al. 2008) |
| CACs | Clathrin adaptor complexes medium subunit family protein | TCTGGGAGAAGAGTGGCTACA / GAGCCACCATTCAAATCCT | 96 | (Marum et al. 2012) |

### 2.4. Physiological and biochemical parameters

#### 2.4.1. Plant water status (water potential and relative water content)

Xylem water potential ($\Psi_{xylem}$) was determined in plants submitted to the drought stress assay, with a Scholander-type pressure chamber (PMS Instrument Co). Measurements were performed at T1, T2, R1 and R2 for both the WW and WS groups.To determine the relative water content (RWC), four leaf discs (diameter = 11 mm) were used per plant (six plants in total) at each point of analysis (T1, T2, R1 and R2). Tissue fresh weight (FW) was recorded and leaf samples were

transferred to tubes with de-ionized water and maintained overnight in dark at 4ºC. On the second day, after carefully removing the excess of water from leaf surface, turgid weight (TW) was registered and leaf discs were dried at 80 ºC for 72 h. After drying, tissue discs were reweighed and dry weight (DW) recorded. RWC was calculated using the following equation: RWC (%) = (FW-DW) / (TW-DW) *100

### 2.4.2. Chlorophyll fluorescence

Chlorophyll fluorescence was determined in expanded leaves of six plants using a portable modulated fluorometer Mini-PAM, including the leaf clip holder part 2030-B (Heinz Walz GmbH) as described in Peña-Rojas et al. (2004). Light-adapted components of chlorophyll fluorescence were measured: steady-state fluorescence (F), maximum fluorescence ($F'_m$) and quantum yield of PSII photochemistry ($\Phi_{PSII}$) equivalent to ($F'_m$ – F) / $F'_m$. Leaves were then dark-adapted for at least 20 min in order to obtain $F_0$ (minimum fluorescence), $F_m$ (maximum fluorescence), $F_v$ variable fluorescence (equivalent to $F_m$ – $F_0$) and maximum quantum yield of PSII photochemistry ($F_v/F_m$).

### 2.4.3. Lipid peroxidation

Lipid peroxidation was estimated by determining the malondialdehyde (MDA) content in leaves according to Elkahoui et al. (2005). Briefly, samples of 100 mg frozen leaves were homogenized in 5 mL of 0.1 % trichloroacetic acid. The homogenates were centrifuged (10 000 × g) and 0.3 mL aliquots of supernatant were added to 1.2 mL of 0.5 % (w/v) thiobarbituric acid prepared in trichloroacetic acid 20 %, and incubated at 95°C for 30 min. After stopping the reaction on ice, samples were centrifuged at 10 000 × g for 10 min at 25 °C. Using a spectrophotometer Genesys 10-uv S (Thermo Fisher Scientific), the supernatant absorbance at 532 nm was measured. After subtracting the nonspecific absorbance at 600 nm, MDA concentration was determined using the extinction coefficient 155 $mM^{-1}cm^{-1}$.

### 2.4.4. Proline

Proline content was assessed as described by Khedr et al. (2003). Briefly, frozen leaves (100 mg) were homogenized in 1.5 mL 3 % (w/v) sulphosalicylic acid and centrifuged (10 000 x g). Two mL of glacial acetic acid and 2 mL of ninhydrin acid were added to the supernatant (100 µL) followed by an incubation of 1 h at 100 °C. After cooling on ice, 1 mL of toluene was added to the reaction mixture. The chromophore phase, containing toluene, was warmed to room temperature and its absorbance was measured at 520 nm using a spectrophotometer Genesys 10-uv S (Thermo Fisher Scientific). The amount of proline was determined from a standard curve ($R^2$= 0.99).

### 2.5. Statistical analysis

Data were compared with SigmaStat software version 3.1 for Windows (SPSS Inc.) by performing One Way analysis of variance (ANOVA) followed by the *post-hoc* Holm-Sidak test (versus control group) when differences existed. In heat treatment, data from stress and recovery periods were compared with results from 25 ºC or R25 ºC, respectively. In drought assay, comparisons were performed against the results from the control group (WW) collected at the same time point. Some data ($\Psi_{xylem}$ of drought stress and proline content of thermal assay) were previously transformed in order to meet the requirements of normality and homogeneity of variances. Differences were considered significant at $P < 0.05$.

## 3. Results

### 3.1. Drought stress

After one-month of drought, no morphological signs of stress were observed (data not shown). However, the results of the RWC (Fig. 1) and $\Psi_{xylem}$ (Fig. 2) showed that the plant water status changed during this period, thereby confirming the physiological stress condition. Leaf water status measured by RWC displayed a significant decrease at T1 and reached 70.9 % at T2 in comparison with 89.6 % determined in WW plants. The $\Psi_{xylem}$, in turn, showed the lowest value at T1 (-1.69 MPa) while at T2 this value increased for -0.78 MPa, being still lower than the one measured in control plants (-0.32 MPa). Following re-watering, the water stress (WS) plants rapidly recovered and displayed similar values to the ones of WW group for both parameters. Results from both chlorophyll fluorescence related parameters ($F_v/F_m$ and $\Phi_{PSII}$) (Table 3) did not show significant differences between water treatments throughout the entire assay, including recovery. The same profile was obtained for proline content (Fig. 3), which was maintained constant in WW and WS plants during drought and recovery. On the other hand, MDA content (Fig. 4) was affected by water deficit, showing a significant increase at T2 (63.6 nmol $g^{-1}$ FW) compared to control plants (32.0 nmol $g^{-1}$ FW). After one day of recovery, MDA levels in WS plants continued significantly higher (49.5 nmol $g^{-1}$ FW) compared to WW plants (37.4 nmol $g^{-1}$ FW), however, these differences disappeared after one week of re-watering.

The relative expression of *QsMYB1.1* and *QsMYB1*.2 transcripts in cork oak stem was not equally affected by the water deficit (Fig. 5). On the day that soil moisture reached 18 % FC (T1), both transcripts were significantly induced in WS plants, namely, 2.4-fold (*QsMYB1.1*) and 5.0-fold (*QsMYB1.2*) compared to WW plants. However, after 30 days at 18 % FC (T2) only the un-spliced variant harboring the 5'UTR intron (*QsMYB1.2*) maintained expression levels significantly higher (1.9-fold) than the corresponding control group. Concerning the recovery period, results showed that one day after re-watering (R1), the relative abundance of *QsMYB1.2* was still 1.9-fold higher in

WS than in control plants. Interestingly, after a week of re-watering (R2), WS plants showed a significant down-regulation of about 1.2-fold and 2.3-fold in the expression levels of *QsMYB1.1* and *QsMYB1.2*, respectively.

### 3.2. Cumulative heat stress

All cork oak plants survived to heat treatment, but on the third day at 55 ºC some stress symptoms were visible in leaves (data not shown). High temperature led to a slight decrease of $F_v$ / $F_m$ (Table 3) reaching the minimum at 55 °C (0.831) that returned to values of control group (R25°C) after recovery. Regarding the $\Phi_{PSII}$ (Table 3) it exhibited a significant increase at 45 °C (0.374) but decayed at 55 ºC (0.185) to values close to the ones before stress (25 ºC, 0.225). RWC was maintained constant throughout the assay (Fig. 1). Free proline determination (Fig. 3) revealed that temperature caused an accumulation of this osmoprotectant solute, that reached a significant high level (0.053 mg $g^{-1}$ FW) at maximum stress (55ºC) when compared to levels at 25 ºC (0.019 mg $g^{-1}$ FW). After one-month recovery, proline content of R25 ºC and R55 ºC plants did not show significant differences. Although MDA content (Fig. 4) exhibited some variation during the time course of experiment, namely an increase at 45 ºC followed by a decrease at 55ºC, these changes were not statistically different from control.

The analysis of relative expression of *QsMYB1* transcripts in stem of cork oaks exposed to heat stress revealed that a gradual and cumulative increase of temperature until 55 ºC led to a decrease on the abundances of both splicing variants (Fig. 5). The expression of *QsMYB1.1* variant was the most affected by high temperature and its abundance started to decrease significantly 1.9-fold at 35 ºC and declined up to 4.0-fold at 55 ºC comparing with levels at 25 ºC. The *QsMYB1.2* relative expression also showed to be modulated by high temperatures, however, it only started to be repressed when temperature reached 45 ºC, showing a slight decay of 1.7-fold at this temperature and 1.9-fold at 55 ºC. After one-month of recovery, *QsMYB1.1* expression levels returned to the control ones while *QsMYB1.2* was still down-regulated and showed an expression 1.7-fold lower than control group.

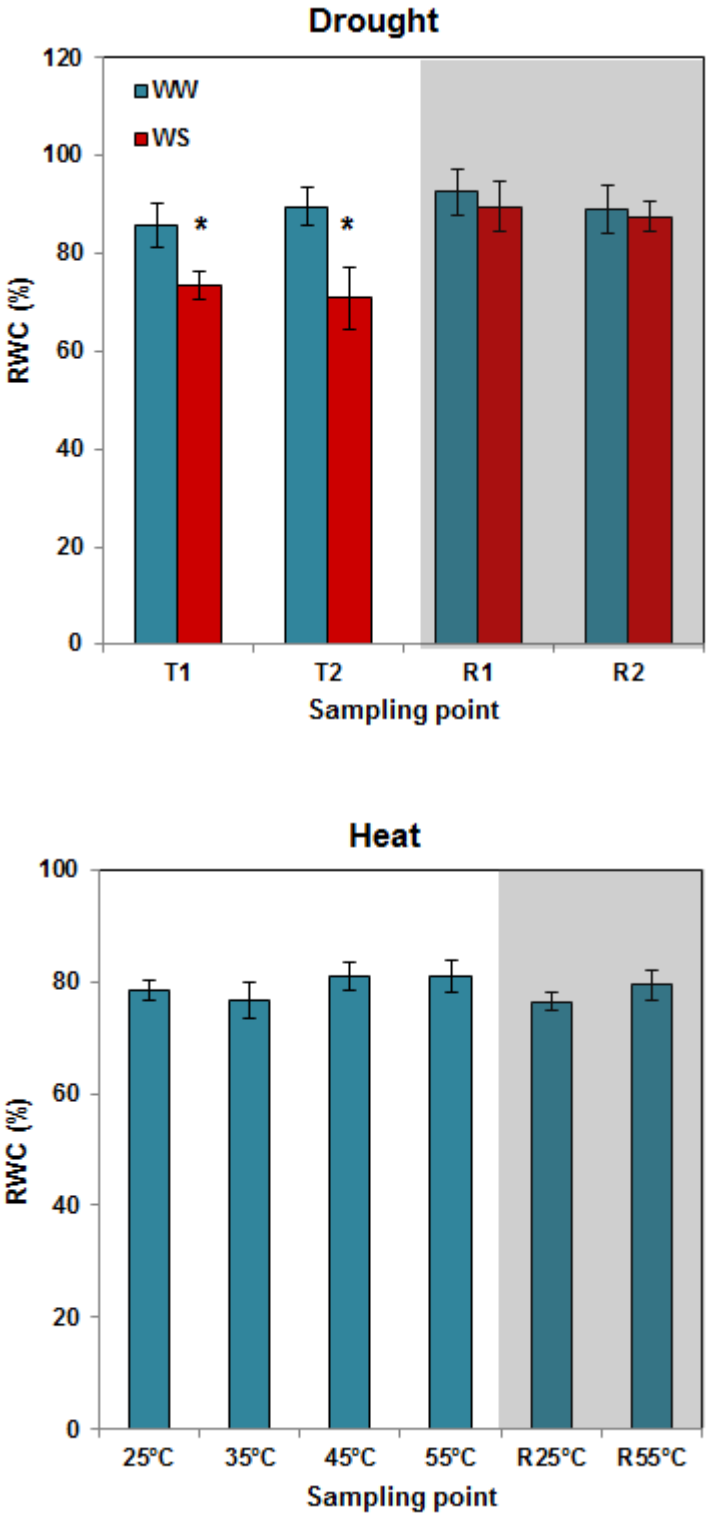


**Fig. 1.** RWC of leaves from cork oaks plants exposed to drought or cumulative heat stress and during recovery. Shaded area marks the recovery period. Data are mean ± SD (n= 6). Asterisks (*) denote statistically different values compared to the corresponding control group (according to Holm-Sidak test, $P < 0.05$).

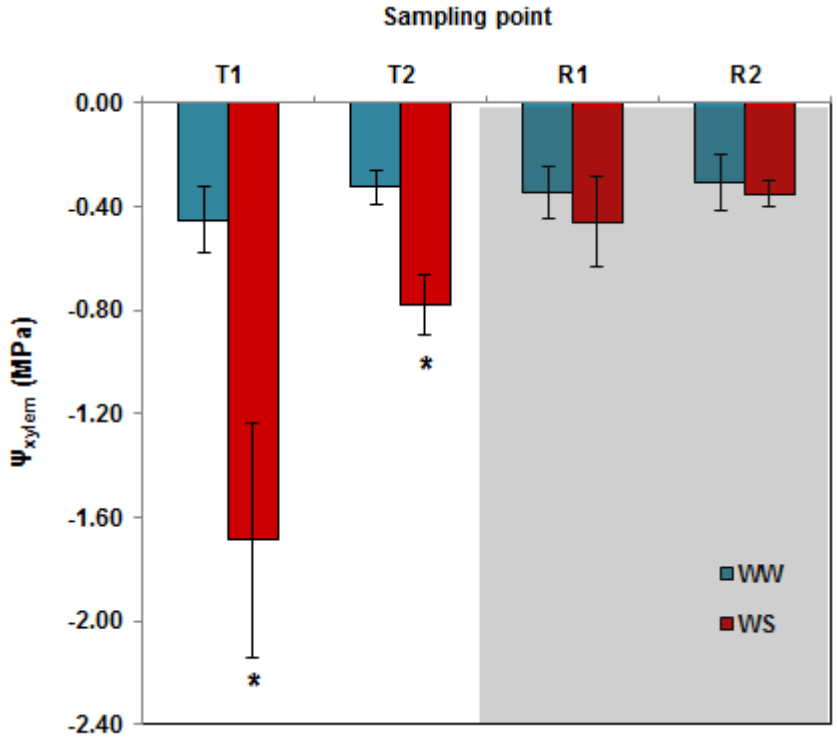


**Fig. 2.** $\Psi_{xylem}$ from cork oak plants exposed to drought stress and during recovery. Shaded area marks the recovery period. Data are mean ± SD (n= 6). Asterisks (*) denote statistically different values compared to the corresponding control group (according to Holm-Sidak test, P < 0.05).

**Table 3.** $F_v/F_m$ and $\Phi_{PSII}$ values of cork oak plants exposed to drought or cumulative heat stresses and during recovery. Data are mean ± SD (n= 6). Asterisks (*) denote statistically different values compared to the corresponding control group (according to Holm-Sidak test, P < 0.05).

| Stress | Sampling point | $F_v/F_m$ | $\Phi_{PSII}$ |
|---|---|---|---|
| **Drought** | T1ww | 0.803 ± 0.056 | 0.750 ± 0.022 |
| | T1ws | 0.811 ± 0.020 | 0.720 ± 0.069 |
| | T2ww | 0.806 ± 0.005 | 0.722 ± 0.051 |
| | T2ws | 0.803 ± 0.024 | 0.735 ± 0.044 |
| | R1ww | 0.805 ± 0.010 | 0.737 ± 0.012 |
| | R1ws | 0.798 ± 0.028 | 0.707 ± 0.022 |
| | R2ww | 0.800 ± 0.007 | 0.750 ± 0.019 |
| | R2ws | 0.813 ± 0.016 | 0.754 ± 0.019 |
| **Heat** | 25 ºC | 0.877 ± 0.005 | 0.225 ± 0.021 |
| | 35 ºC | 0.855 ± 0.012* | 0.276 ± 0.043 |
| | 45 ºC | 0.851 ± 0.010* | 0.374 ± 0.040* |
| | 55 ºC | 0.831 ± 0.012* | 0.185 ± 0.020 |
| | R25 ºC | 0.861 ± 0.009 | 0.270 ± 0.043 |
| | R55 ºC | 0.864 ± 0.006 | 0.263 ± 0.029 |

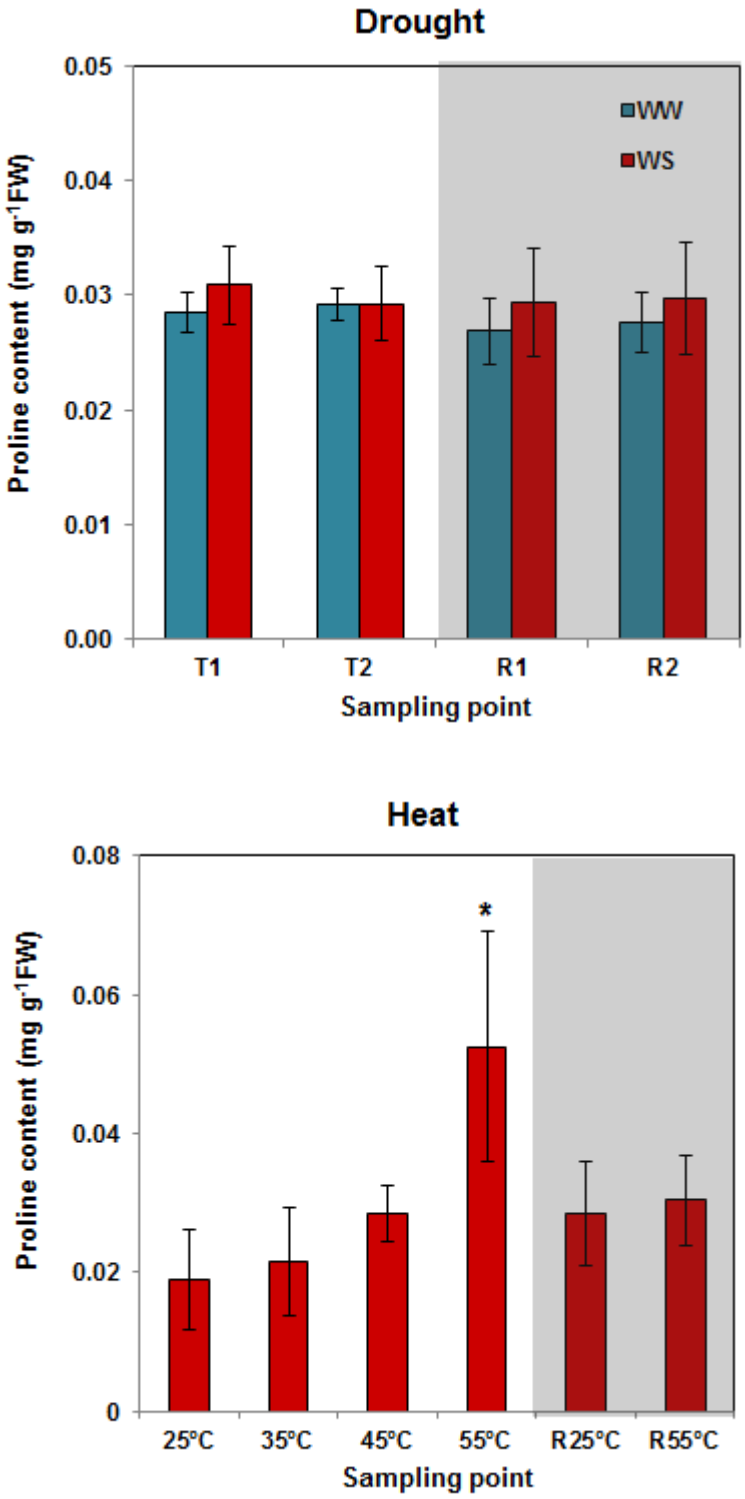


**Fig. 3.** Proline content of cork oak plants exposed to drought or cumulative heat stress and during recovery. Shaded area marks the recovery period. Data are mean ± SD (n= 6). Asterisks (*) denote statistically different values compared to the corresponding control group (according to Holm-Sidak test, $P < 0.05$).

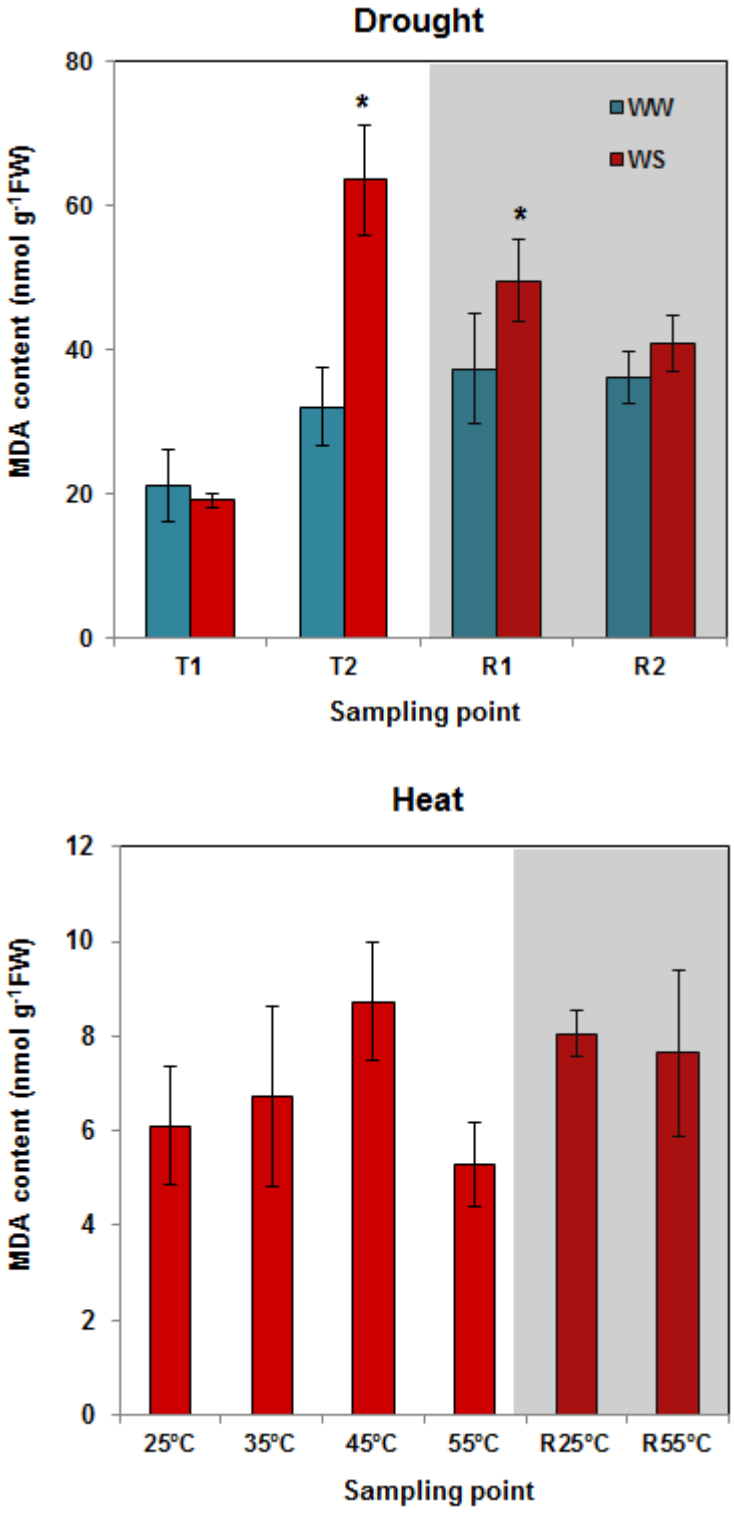


**Fig. 4.** MDA content of cork oak plants exposed to drought or cumulative heat stress and during recovery. Shaded area marks the recovery period. Data are mean ± SD (n= 6). Asterisks (*) denote statistically different values compared to the corresponding control group (according to Holm-Sidak test, $P < 0.05$).

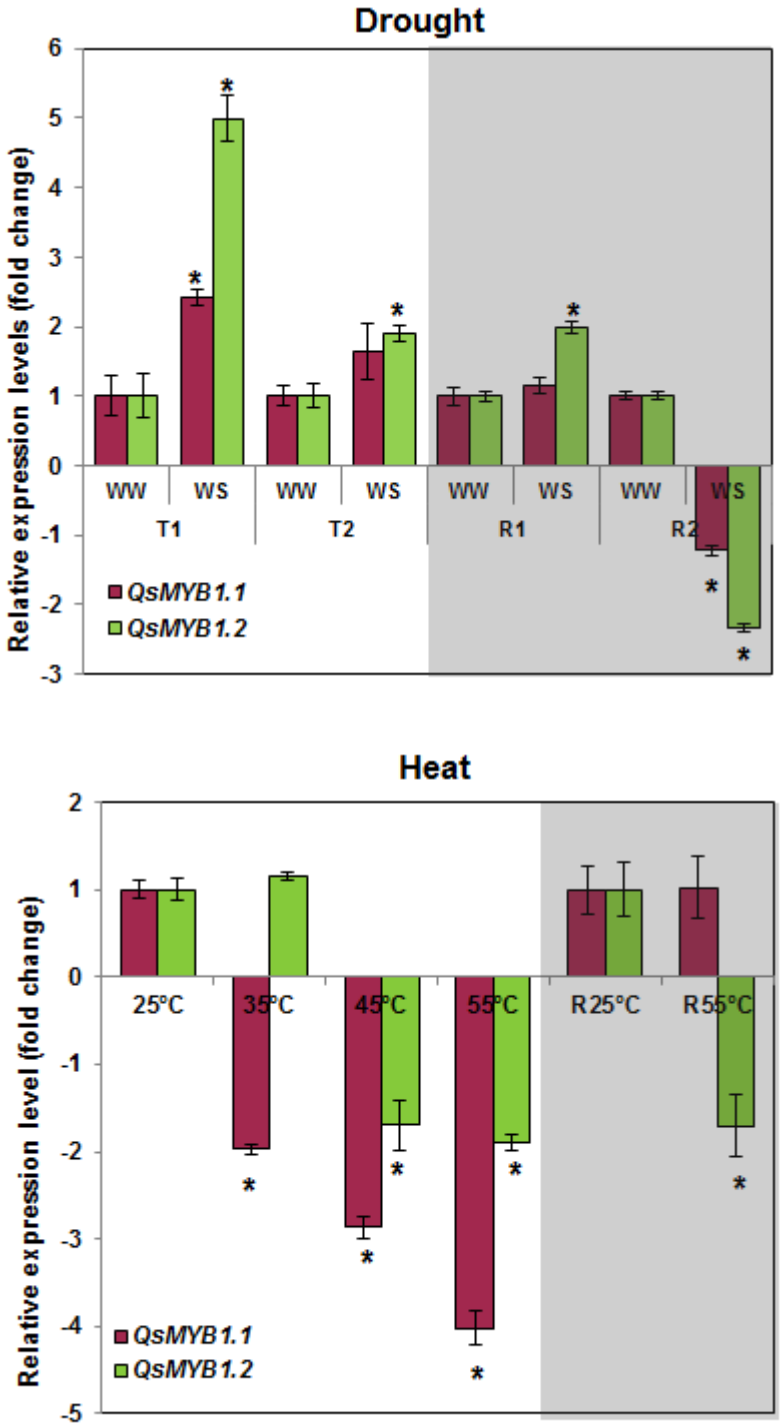


**Fig. 5.** Expression pattern of *QsMYB1* transcripts in response to drought or cumulative heat stress and during recovery. Relative expression levels were assessed by RT-qPCR and are represented as fold change to respective control at each sampling point. *CACs* and *β-TUB* genes were used as reference genes in drought and heat stress condition, respectively. Control groups (T1WW, T2WW, R1WW, R2WW, 25 ºC and R25 ºC) were set as one. Shaded area marks the recovery period. Data are means ± SD (n=2). Asterisks (*) denote statistically different values compared to the corresponding control group (according to Holm-Sidak test, $P < 0.05$).

## 4. Discussion

Cork oak is well-adapted to Mediterranean climate and shows a great plasticity to the characteristic hot and dry summers of this region (Ramírez-Valiente et al. 2010), but the knowledge about the mechanisms underlying this notable stress tolerance is still scarce. Perception of stress stimuli and signal transduction to switch on adaptive responses are the key steps determining plant tolerance to adverse environments (Chinnusamy et al. 2004). Several R2R3-MYB TFs have been associated to abiotic stress response. Here, it was evaluated if the *QsMYB1* TF was regulated at the transcriptional level by heat and drought stresses and thus if it could be considered as a putative player in the transcriptional regulatory network of cork oak under abiotic stresses.

The drought assay revealed that *QsMYB1* transcripts were transiently induced at T1, mainly the *QsMYB1.2* variant. However, after one month at 18 % FC (T2), these differences in transcript abundance (WS versus WW plants) were reduced. This is not a surprising result considering that QsMYB1 as a TF is expected to be activated shortly after the plant has perceived the stress condition in order to regulate the expression of downstream genes, whose products may be involved in the plant response to the adverse environment. Similar results were previously reported by other authors regarding TFs that are highly and transiently induced at the initial period of stress and then decrease their transcript abundances (Ji et al. 2012, Muñiz-García et al. 2012, Ying et al. 2012). The higher value observed for the $\Psi_{xylem}$ at T2 compared to T1 is also supporting this result, suggesting plant adaptation to the water deficit condition, which was kept constant (18 % FC) between these two time-points. However the accumulation of MDA (a marker for oxidative stress) at T2 confirmed the occurrence of stress damages, while the unchanging profile in proline content suggests that this osmoprotectant is not involved in the tolerance to this particular level of stress. In a previous work, Kwack et al. (2011) obtained a similar result when exposed cork oak seedlings to water stress and only detected an increase in proline levels under severe stress (7 - 10 % FC) conditions. However, different results were obtained by Oufir et al. (2009) and Hu et al. (2013) who observed an increase in proline levels after exposure of oak species to drought. This discrepancy in results can be attributed to, for example, different drought conditions or an oak-specific response. Fluorescence parameters did not change throughout the entire assay, indicating that this level of water stress had no impact on photochemical performance. A recent study also revealed that *Quercus pubescens* exposed to soil drought preserved the functionality of the photosynthetic apparatus and controlled the antioxidant system response (Contran et al. 2013). On the other hand, Arend et al. (2013) observed a down-regulation of PSII photochemistry ($F_v$ / $F_m$) and a decrease in chlorophyll content after exposure of *Quercus robur* to drought. Interestingly, after one week of re-watering (R2), there was a slight down-regulation of both transcripts and once more, this effect was more evident on the *QsMYB1.2* expression. Despite physiological parameters indicated that plants had recovered after one week, it seems that at the transcriptional level the re-hydration exerted a regulatory effect on QsMYB1 transcripts. This was already reported by Oono et al. (2003) who identified many genes that were drought-inducible and at same time repressed by

re-hydration using a full-length cDNA microarray from *Arabidopsis*. These authors suggested that both transcriptional and post-transcriptional regulatory systems may be involved in this regulation.

The cumulative effects of heat stress (up to 55 ºC) were clearly visible in terms of expression analysis, since an incremental down-regulation of *QsMYB1* transcripts was observed with temperature rise, showing that plant perceived the increasing stress stimulus and maintained switched on the regulatory response in order to cope with the stressful conditions. It is also noteworthy that *QsMYB1.2* was repressed at 45 ºC and 55 ºC, while *QsMYB1.1* was down-regulated once temperature reached 35 ºC, indicating that each transcript is regulated by different stress signal intensity.

Correia et al. (2013) in a previous work have also exposed cork oak plants to a cumulative heat stress (25 ºC up to 55 ºC) and their electrolyte leakage results suggest that cork oak has the capacity to acclimate even at a high temperature such as 55 ºC. Here, the increasing proline level at 55 ºC suggests that this amino acid can be related to the thermotolerance of *Q. suber*. Results also showed that the photosynthetic performance was affected, especially the $F_v/F_m$ which suffered a slight decrease in comparison to control. However, the observed values are within the range of the ones obtained in healthy plants (Schreiber et al. 1994). After one-month in recovery, the response of both transcripts was different and only the *QsMYB1.1* returned to control values while *QsMYB1.2* transcript remained down-regulated. This may be the result of non-heritable changes conferring short-term stress resistance (i.e. acclimation) or heritable epigenetic modifications responsible for long-term resistance or even transgenerational stress memory (Chinnusamy et al. 2009). Therefore, it would be interesting to further explore these hypotheses.

The response to drought and heat stress has in common the fact that the ratio of *QsMYB1* transcripts varies throughout the assay although both variants are always expressed in simultaneous even in the absence of stress. Previously, other works have already reported the differential alternative splicing pattern in response to various abiotic stresses in several species (Egawa et al. 2006, Qin et al. 2007, Matsukura et al. 2010). But, in most cases, many splicing variants are only induced when plant is exposed to the environmental stress and do not appear in non-stress conditions (Mastrangelo et al. 2012). From our data, it is evident that high temperature has more influence on the regulation of the spliced transcript (*QsMYB1.1*), while drought affects mainly the un-spliced one (*QsMYB1.2*).

In conclusion, our findings showed that both drought and heat stresses followed by recovery have triggered changes on the expression profile of the two *QsMYB1* splicing variants indicating that the QsMYB1 TF is modulated at the transcriptional level by these two abiotic stresses. These conclusions are reinforced by the presence of the so-called heat stress element (HSE, 5'AGAAnnnTTCT3') (Nover et al. 2006) and the drought responsive element (DRE/CRT, GCCGAC) (Egawa et al. 2006) in the *QsMYB1* putative promoter (GenBank accession number JN003628). In addition, the *AtMYB68,* the closest homolog of QsMYB1 in *Arabidopsis* was already described as being induced by high temperature and involved in heat tolerance (Feng et al. 2004). As far as we know this is the first report that identifies a TF with a putative function in the regulatory

network of cork oak response to abiotic stress and recovery, which provides relevant information for further functional studies in order to evaluate the potential of using the QsMYB1 TF in *Q. suber* drought or thermo-tolerance improvement.

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## Supplemental data

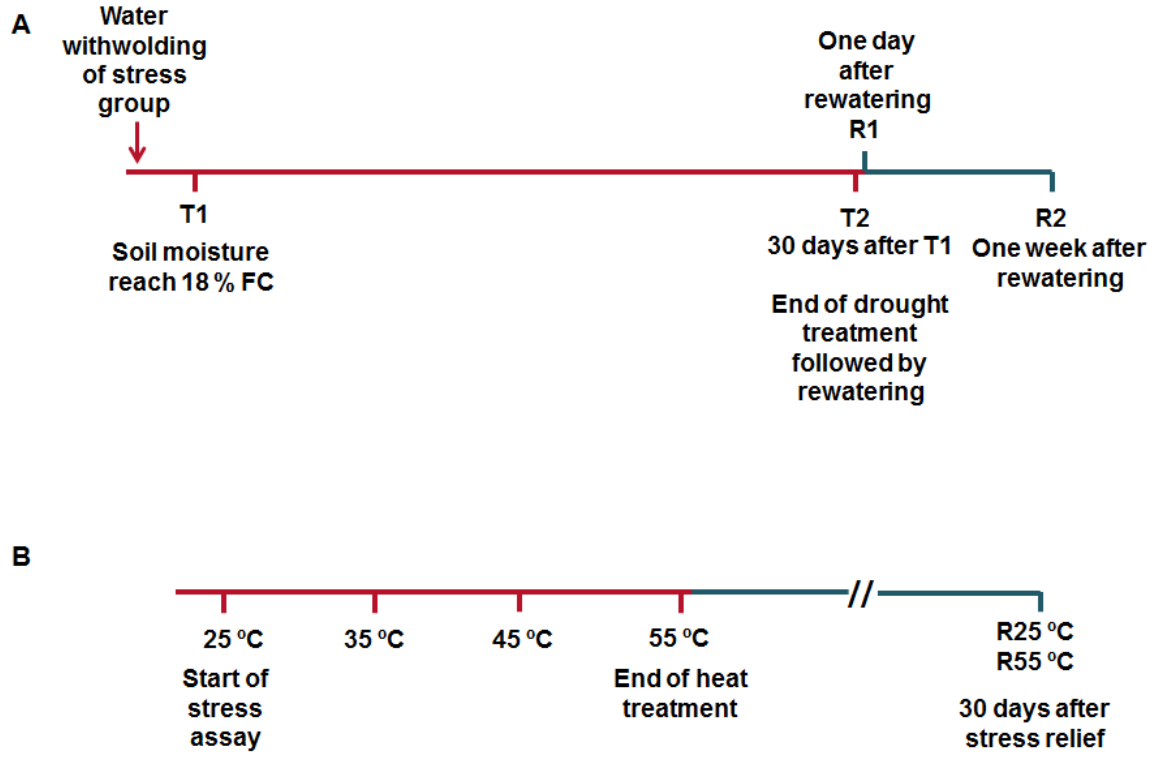


**Fig. S1.** Scheme of drought (A) and cumulative heat stress (B) experiments showing the points of sample collection. Stress period is represented as a red line while recovery is in blue.

# CHAPTER IV

# Effect of exogenous phytohormones on *QsMYB1* expression in cork oak stem

*Chapter to be submitted as a short communication in a SCI journal:*

Almeida T, Pinto G, Santos C, Gonçalves S. Effect of exogenous phytohormones on *QsMYB1* expression in cork oak stem (in preparation).

## Abstract

Phytohormones are key components of plant growth and development that have the ability of inducing changes in plant's transcriptome. However, the knowledge of the molecular mechanisms through which phytohormones act is still limited. The mechanisms behind hormonal control of cork cambium activity and cork development are even scarcer. Among the hormone-responsive genes, transcription factors (TFs) are in a pivotal position. Here, it was evaluated if the expression of an R2R3-MYB TF, the QsMYB1, would be affected by exogenous application of phytohormones, namely abscisic acid (ABA), Methyl jasmonate (MeJa) and indole-3-acetic acid (IAA). *QsMYB1* was previously identified (Chapter II) as being up-regulated in cork-tissues and having hormone-responsive *cis*-acting elements in the putative promoter region. It was also found that the transcription of *QsMYB1* resulted in two splicing variants differing only in the 5'UTR (*QsMYB1.1* and *QsMYB1.2*). The expression analysis by real-time PCR showed that, at least under these experimental conditions, the expression of *QsMYB1* transcripts was only weakly changed by ABA; MeJa early induced the expression of *QsMYB1.2* transcript, suggesting that QsMYB1 may integrate MeJa signal transduction; IAA treatment resulted in a complex expression pattern of both transcripts that need additional studies.

***Keywords*:** R2R3-MYB, transcription factor, cork, abscisic acid, methyl jasmonate, indole-3-acetic acid, gene expression

***Abbreviattions:*** ABA, abscisic acid; ACT, actin; CACs, clathrin adaptor complexes medium subunit family protein; $C_T$, threshold cycles; EF-1α, polymerase elongation factor-1α; IAA, indole-3-acetic acid; JA, jasmonic acid; MeJa, Methyl jasmonate; PAT, polar auxin transport; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; TFs, transcription factors.

## 1. Introduction

Plant hormones or phytohormones are organic molecules synthesized by plants that act at low concentrations (Aloni et al. 2007). Several types of phytohormones have been discovered, such as auxin, cytokinin, gibberellins, abscisic acid (ABA), ethylene, brassinosteroids, jasmonic acid (JA) and salycilic acid (Jiang and Guo 2010). Phytohormones are known to play key roles in plant growth and development as well as in response to biotic and abiotic stresses by regulating genome expression. The action of hormones is mediated by signal transduction cascades (Wang and Irving 2011) exhibiting extensive crosstalk and signal integration with each other and with environmental and developmental signaling pathways (Gray 2004). Among the hormone-responsive genes, transcription factors (TFs) are in a prominent position since they can regulate the expression of several other genes whose resulting proteins will have a great impact on how the plant react and adapt to the external stimuli. MYB genes with a two-repeat R2R3 DNA binding domain constitute the largest MYB gene family in plants (Stracke et al. 2001). Numerous R2R3-MYB proteins have been characterized and found to be associated with several plant-specific processes including (1) control of cell morphogenesis, (2) response to biotic and abiotic stresses, (3) phenylpropanoid biosynthetic pathway and (4) response to phytohormones (reviewed in Du et al. 2009, Dubos et al. 2010). In a previous work we reported the molecular characterization of *QsMYB1* gene encoding for an R2R3-MYB TF from cork oak (*Quercus suber* L.) that was preferentially expressed in cork tissues suggesting its possible involvement in the regulatory network of cork biosynthesis (Almeida et al. 2013a). Two splicing variants (*QsMYB1.1* and *QsMYB1.2*), differing only in the 5'-untranslated region, were identified and the in silico analysis of the putative promoter region revealed the presence of various *cis*-acting elements related to stress and hormone responses.

Cork results from the activity of the cork cambium (or phellogen) and is a protective tissue of suberized dead cells that separates the living cells of the plant from the outside environment (Silva et al. 2005, Pereira 2007). When the cork layer is removed, as a wound-healing response, a traumatic phellogen differentiates showing an enhanced meristematic activity in the following years (Graça and Pereira 2004, Pereira 2007). Climatic conditions (e.g. temperature and drought) are among the multiple factors that can influence cork growth and consequently its quality. Usually, in the plant response to an adverse condition, the signaling starts with perception of a hormone by a receptor and continues with the proliferation of the signal, eventually leading to changes in gene expression (Zubo et al. 2011). The hormonal regulation of vascular cambium development and wood formation has been quite explored (Serh et al. 2010, Hou et al. 2006, Nilsson et al. 2008). However, the available data on the hormonal control of cork cambium activity and cork development is very scarce (Soler et al. 2007). In this work it was evaluate if the expression of *QsMYB1* would be affected by exogenous application of phytohormones, namely ABA, Methyl jasmonate (MeJa) and indole-3-acetic acid (IAA).

ABA, also known as the stress hormone, has been shown to regulate many aspects of plant development in response to unfavorable environmental stresses, allowing the plant to cope and

survive in adverse conditions, such as drought, low or high temperature, or high salinity (Hong et al. 2013). This stress mediator is implicated in many physiological processes such as seed maturation and dormancy, inhibition of seed germination, stomatal closure and abscission (Assman 2010, Wang and Irving 2011). Furthermore, ABA is also likely involved in slowing down and stopping wood formation in trees towards their winter dormancy by retarding and ending their cambium activity (Aloni 2007). ABA has also been described as being involved in the regulation of wound-induced suberization in potato wound periderm (Lulai et al. 2008). In a recent genomic approach to cork biosynthesis, Soler et al. (2007) identified (in phellem) an up-regulated gene similar to an *Arabidopsis* annexin that senses the $Ca^{2+}$ signal elicited by ABA, suggesting that ABA may be also involved in the regulation of cork development

MeJa is a volatile methyl ester derived from the JA and has been considered an important candidate for an airborne signal molecule mediating intra- and interplant communications, modulating plant defence responses to insect-driven wounding, pathogens and environmental stresses (Cheong and Choi 2003, Avanci et al. 2010). In addition, jasmonates (MeJa and JA) have been described as cellular regulators acting in diverse developmental processes such as germination, root growth, fertility, fruit ripening and senescence (Cheong and Choi 2003). MeJa is also involved in the regulation of the biosynthesis of several secondary metabolites including terpenoids, alcaloids, phenylpropanoids and antioxidants (Avanci et al. 2010). For instances, exogenous application of MeJa in needles of white spruce resulted in an accumulation of the chalcone synthase transcript, which catalyses the first step of flavonoid biosynthesis pathway (Richard et al. 2000).

IAA is the most abundant natural form of auxins in plants (Aloni 2007). It is synthesized from actively growing tissues such as shoot meristems, leaf primordia, young expanding leaves, developing seeds, fruits and pollens (Wang and Irving 2011). Two main pathways describe the transport of auxin, a fast, non-directional transport in phloem and a slower, directional, so-called polar auxin transport (PAT) in various tissues (Friml and Palme 2002). This hormone is a key regulator of a wide range of developmental processes, including initiation and elaboration of final morphology of leaves and vascular network (reviewed in Scarpella et al. 2010), wood formation (Nilsson et al. 2008), apical dominance (Müller and Leyser 2011), lateral root development (Casimiro et al. 2001) and first periderm formation in woody stems (Lev-Ladun and Aloni 1990).

Several studies have shown that some members of the R2R3-MYB family are responsive to phytohormones. For instance, the MYB77 from *Arabidopsis* is a component in auxin signal transduction and modulates lateral root growth. In addition, MYB77 interacts with auxin responsive factors (ARFs) to promote auxin-responsive genes expression (Shin et al. 2007). In tobacco, the NtMYBJS1 is MeJa inducible and activates several early phenylpropanoid-related genes (Gális et al. 2006). Other example is the MYB96 from *Arabidopsis* that regulates drought stress response by integrating ABA and auxin signals (Seo et al. 2009).

The putative function of QsMYB1 TF in the regulatory network of cork formation together with the presence of hormonal-responsive *cis*-acting elements in its putative promoter (chapter II, Fig.

2), led us to the present work aiming to provide the first insight on the regulation of *QsMYB1* expression by exogenous phytohormones. Therefore, here, the goal is to evaluate if QsMYB1 can be considered as a candidate component in hormone signal transduction during cork development.

## 2. Material and methods

### 2.1. Plant growth conditions and hormone treatments

Seed grown twelve-month-old cork oak plants were acquired from a forest plant producer (Portugal) and transferred from semi controlled greenhouse conditions to a climate chamber for a 2 weeks acclimation period. During this period, the climate chamber environment was kept constant at 25 ºC / 20 ºC (day / night) of air temperature, 60-70% relative humidity, 250 µmol $m^{-2}$ $s^{-1}$ of photosynthetic photon flux, 16 h light photoperiod and plants were watered daily. All hormones were purchased from Duchefa Biochemie.

Plant treatments with hormones ABA and MeJa were applied over leaves and stems with a handheld sprayer. ABA treatment consisted in spraying a 100 µM ABA solution in 0.1% v/v ethanol. A control group was sprayed with 0.1% v/v ethanol in distilled water without ABA. Stem samples were harvested at 1, 2, 4, 8, 24 and 48 h after treatment. Plants were always kept in the growth chamber. MeJa was applied by spraying a 22 mM MeJa solution suspended in 2.5% v/v ethanol. The control was treated only with the carrier solution (2.5 % v/v ethanol in distilled water). To avoid cross-contamination, both the MeJa-induced and the control plants were treated and kept in two separate rooms during the entire assay. Stem samples were taken at 1, 4, 24 and 48h. In order to avoid variation due to biological development, a control sample was collected each day of treatment at same time of treated samples (1h after treatment, 24 and 48h). In both treatments, 2 pools (10 plants each) of stems were harvested randomly from both hormone-induced and control plants at each sampling point.

For IAA treatment, in order to test two different ways of applying IAA, two approaches were conducted, namely, IAA-L and IAA-M. In IAA-L approach, 30 cork oak plants were decapitated at internode 6, defoliated and a sample of 1 cm was cut from each stem (untreated control, D0). After that, lanolin was applied to the decapitated stems. Twenty-hours later, a new 1 cm sample (auxin-depleted, D20) was collected from each stem; Auxin (60 mM) was dissolved in warm ethanol and mixed with melted lanolin as an inert carrier (92 lanolin: 8 IAA/ethanol). After cooling, a drop of approximately 20 µL of IAA-lanolin mixture was then applied to the cut stems and finally, after 4 h, a new 1 cm stem sample (auxin-induced, i4h) was collected from each plant. During incubation periods, the plants were kept in the growth chamber. The IAA-M approach was performed by cutting stem segments of approximately 6 cm (beneath shoot apex, internode 6) from plants previously defoliated. The untreated control sample (D0) was immediately frozen and the remaining stems were immersed in ½ Murashige and Skoog (MS) medium and incubated at room

temperature. Samples were taken after 2 and 20 h (auxin-depleted, D2 and D20). After this 20 h period, 0.6 mM of IAA (final concentration) was added to the MS medium and stems segments were further incubated 0.5, 2 and 4h (auxin-induced, i0.5h, i2h, and i4h) before sampling. In both approaches, 3 pools (10 stem segments each) were sampled at every time-point. After collection, stem samples were immediately frozen in liquid nitrogen and stored at -80 ºC until RNA extraction.

**2.2. RNA extraction, reverse-transcription quantitative polymerase chain reaction (RT-qPCR)**

Total RNA was isolated from stem samples using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) following manufacturer's instructions with minor modifications: addition of 50 µL from Plant RNA Aid solution (Ambion) to the lysis buffer and the use of 64% (v:v) ethanol instead of binding solution. The protocol also included an optional step to remove genomic DNA (gDNA) using the On-column Dnase I digestion set (Sigma-Aldrich). A single RNA extraction was performed for each pool. At the end, RNA samples corresponding to the same point of analysis were pooled together through a precipitation step with 10 M LiCl followed by washing with 70 % (v/v) ethanol and dissolution in RNase free water. RNA concentration and purity were assessed with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) and the integrity was verified on a 1% (w:v) agarose gel. First-strand cDNAs were synthesized from 1.5 µg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen), which includes an additional genomic DNA elimination step and the use of a mix of oligo(dT) and random hexamer primers. The sequences of all primers used in the RT-qPCR analysis were the same previously described in Chapter III.

*QsMYB1* transcripts were specifically amplified using the primer QsMYB1fwd together with one of the following reverse oligonucleotides: BNDrev to amplify the *QsMYB1.1* transcript (GenBank accession number JF970262) or the SSRrev for the amplification of *QsMYB1.2* transcript (GenBank accession number JF970263). The PCR mixture included 1X iQ™SYBR® Green Supermix (Bio-Rad Laboratories), 0.2 µM of each primer and 2 µL of the previously synthesized cDNA in a final volume of 20 µL. The RT-qPCR programme consisted in an initial incubation at 95°C for 3 min, followed by 40 cycles with 10 s at 95 ºC, 15 s at 57 °C / 60 ºC (SSRrev / BNDrev) and 10 s at 72 °C with a single fluorescent reading taken at the end of each cycle. No template controls (NTC) for each pair of primers were included in each run. Two independent experiments were performed with three technical replicates each. To distinguish specific from nonspecific products and primer dimers, a melting curve was obtained immediately after amplification. PCR and melting products were detected in real time with an iCycler iQ5 Instrument (Bio-Rad Laboratories). The threshold cycles ($C_T$) were calculated by the optical interface iQ5 software (Bio-Rad Laboratories). Absence of gDNA contamination was confirmed by RT-qPCR using a pair of primers (Ex1fwd / Ex2rev) spanning an intron in the coding region. Three reference genes previously described (Soler et al. 2008, Marum et al. 2012) were tested: actin (*ACT*), polymerase elongation factor-1α (*EF*-1α) and clathrin adaptor complexes medium subunit family protein (*CACs*). PCR conditions and cycling parameters were the same as before with an

annealing temperature of 60 °C. The same batch of cDNA was used on the amplification of target and reference genes. According to Normfinder (Anderson et al. 2004) results (Table 1), *ACT* gene was chosen as the most stable during MeJa induction, *EF-1α* showed to be more stable during ABA and IAA-M treatments while in the IAA-L approach was used the *CACs* as reference gene to normalize samples. Amplification efficiencies (E) of primers were determined using standard curves with a 10-fold dilution series, giving E values close to 100 %. Relative abundances of *QsMYB1* transcripts were calculated by the comparative $2^{-\Delta\Delta Ct}$ method (Livak and Schmittgen 2001). Expression levels are represented as fold change relative to the respective control group in each sampling point.

**Table 1.** Stability values of candidate reference genes calculated by the Normfinder algorithm using linearized expression data from stem cDNA samples. For each treatment, it was chosen the candidate gene showing the lowest stability value (in bold) to normalize samples.

| Hormone treatment | Reference gene | Stability value |
|---|---|---|
| **MeJa** | EF-1α | 0.190 |
| | ACT | **0.065** |
| | CACs | 0.185 |
| **ABA** | EF-1α | **0.042** |
| | ACT | 0.151 |
| | CACs | 0.122 |
| **IAA-L** | EF-1α | 0.356 |
| | ACT | 0.256 |
| | CACs | **0.124** |
| **IAA-M** | EF-1α | **0.016** |
| | ACT | 0.047 |
| | CACs | 0.087 |

### 2.3. Statistical analysis

Data were compared with SigmaStat software version 3.1 for Windows (SPSS Inc.) by performing One Way analysis of variance (ANOVA) followed by the *post-hoc* Holm-Sidak test (versus control group) when differences existed. Differences were considered to be statistically significant at $P \leq 0.05$.

## 3. Results and discussion

In order to perform a preliminary evaluation of the effects of phytohormones on the transcriptional regulation of *QsMYB1*, three phytohormones (ABA, MeJa and IAA) were exogenously applied to cork oak plants, and the expression pattern of *QsMYB1* AS variants in stems was analyzed by RT-qPCR (Fig. 1 and Fig. 2). After application of ABA (100 µM), the analysis showed that there was no significant changes in the expression of *QsMYB1* variants in the first 8 h. But, 24 h upon ABA treatment a weak down-regulation of 1.56-fold (*QsMYB1.1*) and 1.17-fold (*QsMYB1.2),* comparing with control (24h-ctrl), was observed. After 48 h, the levels of *QsMYB1* transcripts equaled the ones in control (48h-ctrl). Therefore, after ABA induction the changes in *QsMYB1* abundances were lower than 2-fold. This suggests that, at least under these experimental conditions, ABA had no great influence on *QsMYB1* transcripts expression. Numerous TFs, including some R2R3-MYB, have been shown to be important regulators of ABA-mediated gene expression, mainly under environmental stress conditions (e.g. drought, cold and high salt) (Tuteja 2007, Fujita et al. 2011). Moreover, some MYC and MYB proteins are known to be synthesized only in a late stage of stress response, after endogenous ABA accumulation (Tuteja 2007). Hence, despite the results, it will be interesting to further study other ABA concentrations and/or extend the time of the assay, since QsMYB1 appears to be involved in cork oak response to abiotic stresses, namely dought (chapter III, Almeida et al. 2013).

Both *QsMYB1* transcripts showed to be differently influenced by MeJa elicitation (Fig. 1). Regarding the *QsMYB1.2* variant, there was an up-regulation of transcript levels in 3.4-fold after 1h and 2.9-fold 4 h after hormone application compared to control (1 h-ctrl). But, at 24 h its abundance returned to normal and at 48 h remained identical to the corresponding control (48h-ctrl). On the other hand, MeJa has little effect on the *QsMYB1.1* relative abundance, which decreased 1.2-fold at 1 h and 1.0-fold at 4 h of hormone treatment, reaching the minimum levels at 24 h (-1.8-fold) and returning to control (48 h-ctrl) values at 48h. Therefore, results suggest that the effect of MeJa is mainly exerted on the un-spliced variant whose mRNA levels were transiently induced as early as 1 h after hormone treatment while the spliced transcript was only weakly repressed. This may indicate that MeJa is putatively modulating the *QsMYB1* AS mechanism. MeJa has been reported as a regulator of the defence-related gene expression (reviewed in Reinbothe et al. 1994). Previous studies have shown that exogenous MeJa is able to elicit genes of phenylpropanoid pathway in several tree species, namely *Taxus chinensis* (Li et al. 2012), *Picea glauca* (Richard et al. 2000) or in *Picea abies* (Galliano et al. 1993). Phenylpropanoid-based polymers like lignin, suberin or condensed tannins are essential to the stability and robustness of plants towards mechanical or environmental damages, like drought or wounding (Vogt 2010). As mentioned before, when cork is removed from the cork oak tree, there is the subsequent differentiation of a traumatic phellogen as a wound-healing response. Indeed, in the work of Soler et al. (2007), authors identified some phellem up-regulated genes encoding for enzymes involved on lipid catabolism, such as LOX1. These enzymes are known to initiate the synthesis of wound hormones

such as jasmonates (Schaller and Stintzi 2008). Thus, the regulation of *QsMYB1* expression by MeJa may be related to this wound-response and this should be explored in further studies.

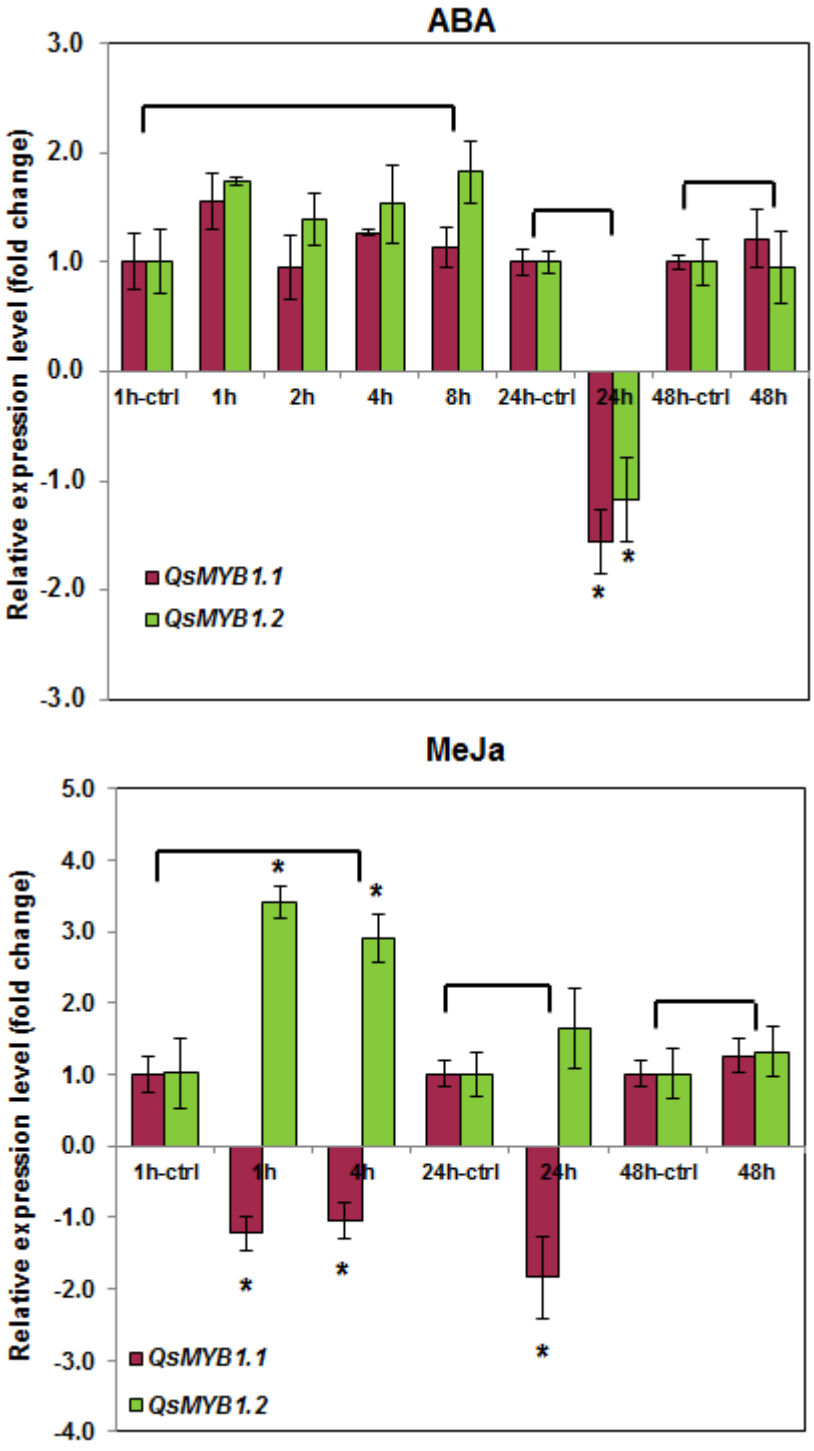


**Fig 1**. Time-course expression of *QsMYB1* AS variants in cork oak stems in response to exogenous ABA (100 µM) and MeJa (22 mM) evaluated by RT-qPCR in cork oak stems. Expression levels are shown as fold change relative to control (1h-ctrl equals 1). Data are means ± SD (n=2). Asterisks (*) denote statistically different values compared to the corresponding control group (according to Holm-Sidak test, $P < 0.05$).

The two approaches for IAA treatment resulted in a similar response by the *QsMYB1* transcripts (Fig. 2). In the IAA-L approach, after 20 h of IAA-depletion, the relative levels of both transcripts declined 4.0- (*QsMYB1.1*) and 3.0-fold (*QsMYB1.2*) compared to the untreated control. Four hours after hormone application, the down-regulation was of 2.7-fold for *QsMYB1.1* and 3.5-fold for *QsMYB1.2* compared to levels at D0. In IAA-M procedure, additional sampling points both in depletion and induction phases were included. The relative levels of *QsMYB1* transcripts suffered a rapid drop of 6.1- (*QsMYB1.1*) and 5.7-fold (*QsMYB1.2*) just 2 h after IAA-depletion initiation. Following 20 h of IAA-depletion, differences between mRNA levels in D20 and D0 samples were reduced, showing a decrease of 3.7- and 3.9-fold in *QsMYB1.1* and *QsMYB1.2*, respectively. But, after hormone addition, transcripts levels decreased again up to 10.9-fold (*QsMYB1.1)* and 10.2-fold (*QsMYB1.2)* at the end of 4h-induction period.

During the depletion phase, a down-regulation of *QsMYB1* transcripts was observed in both approaches. Hence, in the case of *QsMYB1* being auxin-responsive, one would expect an increase in *QsMYB1* transcripts rather than a decrease after IAA addition. Without further studies we can only speculate about some reasons for this result. Both approaches were based on methodology used in previous works with other species (Baba et al. 2011, Schrader et al. 2002, Esmon et al. 2006, Nilsson et al. 2008, Bjöklun et al. 2007). Moreover, several studies focusing on regulation of cambial activity and wood formation have demonstrated that labeled auxin, apically applied to decapitated stems or stem segments, moves in a basipetal pollar fashion (Sundberg and Uggla 1998, Nilsson et al. 2008, Little and Sundberg 1991, Bjöklun et al. 2007). Therefore, we consider that the probability of IAA has not penetrated into the underlying tissues is low, unless for some reason the auxin transport capacity has been lost. Our previous findings showed that *QsMYB1* is preferentially expressed in cork tissues. Thus, one possible explanation for the observed expression pattern, following IAA application, is that cells in cork tissues may have not been exposed to the same auxin levels than xylem cells. Consequently, the IAA levels in cork tissues may have not reached values high enough to trigger the induction of *QsMYB1* transcripts again. Other hypothesis is that the *QsMYB1* may be wounding-responsive and consequently the cut performed in both approaches could be also responsible for the observed down-regulation in transcripts levels. Therefore, the experimental setups used in the two approaches may not be the most adequate to evaluate the effect of auxin on *QsMYB1* gene and, hence, additional studies should be conducted.

In conclusion, this study provides first data regarding the effect of exogenous application of phytohormones on the expression of *QsMYB1* transcripts in cork oak stem. The RT-qPCR analysis suggests that at least under these experimental conditions, the expression of QsMYB1 transcripts was only weakly changed by ABA, whereas MeJa early induced *QsMYB1.2* transcript. On the other hand, treatment with IAA gave rise to a complex expression pattern that needs to be further studied. The results presented here is therefore a start point to delineate more studies that will help to elucidate whether QsMYB1 may be mediating phytohormones signaling during cork biosynthesis.

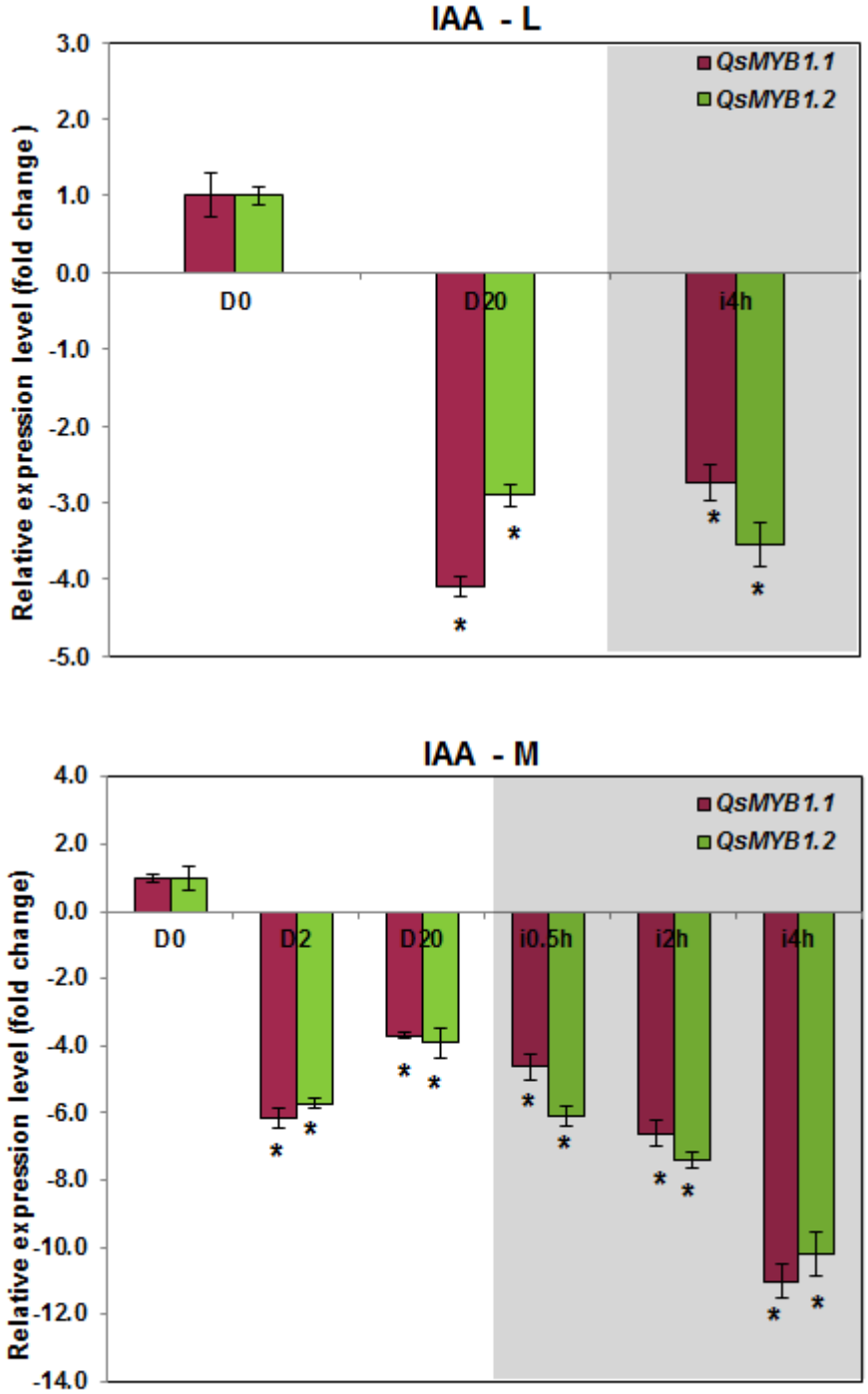


**Fig 2**. Time-course expression of *QsMYB1* AS variants in cork oak stems in response to exogenous IAA applied using two different experimental approaches (IAA-L and IAA-M) evaluated by RT-qPCR. Expression levels are shown as fold change relative to control (D0 equals 1). Shaded area marks the hormone-induction phase. Data are means ± SD (n=2). Asterisks (*) denote statistically different values compared to control group (according to Holm-Sidak test, $P < 0.05$).

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# CHAPTER V

## Overexpression of QsMYB1 in hybrid aspen (*Populus tremula* L. *x tremulóides* Michx.)

## Abstract

The work developed in previous chapters determined the expression profile of QsMYB1 in different cork oak organs and tissues and also the modulation of QsMYB1 expression by abiotic stresses and exogenous hormones. The results have pinpointed putative function(s) of QsMYB1; however, to proceed in the knowledge of this transcription factor and determine the role of QsMYB1 in cork development, functional studies must be developed. Therefore, in this chapter, a reverse genetic approach was initiated, by constitutive overexpression of QsMYB1 in hybrid aspen (*Populus tremula* L. *x tremulóides* Michx.). Several transgenic lines were obtained and the quantification of *QsMYB1* expression level was performed in leaves and stems of transformed hybrid aspen (grown in vitro) by real-time PCR. Three transgenic lines showing different expression levels of *QsMYB1*, both in leaves and stems, were selected for further functional analysis.

***Keywords:*** Cork, R2R3-MYB, overexpression, reverse genetics, Populus, hybrid aspen.

***Abbreviations:*** BAP, 6-benzylaminopurine; $C_T$, threshold cycles; IBA, indole-3-butyric acid; ORF, open reading frame; RT-qPCR, reverse-transcription quantitative polymerase chain reaction; TDZ, thidiazuron; TFs, Transcription factors;

## 1. Introduction

Transcriptional regulation has a major role in the control of gene expression in plants and is crucial for many biological processes (e.g. plant development and response to environmental stimuli) (Mitsuda and Takagi 2010). Transcription factors (TFs) are key players in this regulation since they are able to control promoter strength of their target genes, activating or repressing gene expression (Stracke et al. 2001). Thus, TFs can modulate the transcriptome by controlling one or more genes which in turn leads to metabolic and phenotype changes. Therefore, the manipulation of TFs provides the possibility to induce phenotypic changes more efficiently than the manipulation of other genes (Mitsuda and Takagi 2010).

Expression studies have suggested that QsMYB1 TF may be involved in the regulatory network of cork biosynthesis and two alternative splicing variants were identified (*QsMYB1.1* and *QsMYB1.2*) (Chapter II, Almeida et al. 2013a). Exposure of cork oak plants to cumulative high temperature and drought stresses has also indicated that QsMYB1 TF may be involved in the cork oak response to these two abiotic stresses (Chapter III, Almeida et al. 2013b). In addition, *QsMYB1* seems to be responsive to methyl jasmonate, since its exogenous application in cork oak plants had triggered a transient change in expression of both *QsMYB1* variants (Chapter IV). However, these conclusions are only founded in changes at the transcriptional level. Hence, functional genetic studies are required to confirm its involvement in these plant processes.

Hardwood tree species, in general, and oaks, in particular, have proven to be recalcitrant to genetic transformation (Sánchez et al. 2005). Nevertheless, there are already some works reporting genetic transformation of cork oak (Sánchez et al. 2005, Álvarez and Ordás 2007, Álvarez et al. 2009), but this is still not a straight-forward protocol and its efficiency is very dependent on the bacterial and plant genotype (Álvarez and Ordás 2007). These limitations together with the long reproductive cycle of cork oak do not turn its genetic transformation an attractive strategy for functional studies and therefore other plant systems are commonly used for this purpose. For instance, *Arabidopsis* is a widely used model system to address questions related to secondary growth. However, with the completion of *Populus* genome sequence and the increasing availability of genomic and molecular biology resources for this genus, *Populus* has become a consensus model for woody plant functional genomics since it enables a more detailed and integrated analysis of secondary growth (Dharmawardhana et al. 2010, Jansson and Douglas 2007). *Populus* transformation efficiency is also dependent of *Populus* species and within-species genotypes (Ye et al. 2011), but several protocols have already been established (Nilsson et al. 2002, Song et al. 2006, Yevtushenko and Misra 2010). Comparing with some recalcitrant species, *Populus* transformation is more efficient, since it requires less time, is not as complicated and yields a high number of stable transformation events (Ye et al. 2011).

In this work, *QsMYB1* was constitutively overexpressed in the hybrid aspen (*Populus tremula* L. *x tremulóides* Michx.) heterologous system and transgenic lines, showing different levels of *QsMYB1* expression, were obtained.

## 2. Materials and methods

### 2.1. **Vector construction and hybrid aspen transformation**

For QsMYB1 overexpression, the complete cloned open reading frame (ORF) sequence was sub-cloned into pDONOR221 using the Gateway® recombination (Invitrogen) (Karimi et al. 2002) and sequenced. Then it was recombined with the Gateway® binary expression vector pK7GW2.0 (Fig. 1), sequenced and introduced into *Agrobacterium tumefaciens* (strain GV3101 pMP90) (Koncz and Schell 1986). In parallel, *A. tumefaciens* GV3101 was also transformed with the empty vector without the gene of interest. Hybrid aspen (*Populus tremula* L. *x tremulóides* Michx., wilde type clone T89) was sub-cultured on Murashige and Skoog (MS) basal salt medium at half-strength (Murashige and Skoog 1962) under a cycle of 16h light / 8h dark at 22ºC /18 ºC.

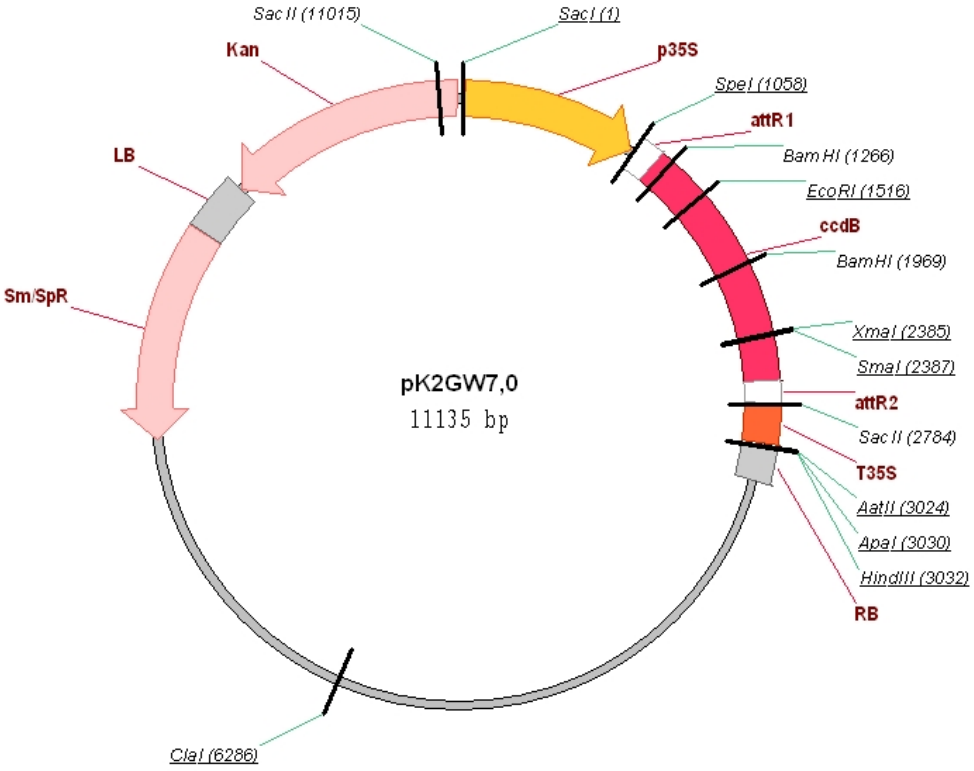


**Fig. 1.** Map of the GATEWAY® binary expression vector used to overexpress QsMYB1 in hybrid aspen**.** attR (recombination sites), ccdB ("killer" gene), LB (left border), Kan (kanamycin resistance gene), p35S (promoter 35S), RB (right border), Sm/SpR (streptomycin/spectinomycin resistence gene), T35S (terminator 35S). http://gateway.psb.ugent.be/vector/show/pK7WG2/search/index/overexpression/any (last acessed November 2013).

Hybrid aspen was transformed according to Nilsson et al. (1992) with minor modifications. Briefly, *A. tumefaciens* transformed either with the overexpression construct (35S::QsMYB1) or empty vector (EV, negative control) was grown on YEB medium supplemented with 50 µg $mL^{-1}$ spectinomycine and 50 µg $mL^{-1}$ rifampicine overnight at 28 ºC. When $OD_{600}$ reached 0.3-0.8, the culture was centrifuge 20 min at 4000 rpm and ressuspended on MS medium (pH 5.8) to a final $OD_{600}$ of 0.6. Then the culture was incubated with 20 µM of acetosyringone at room temperature (RT) during 1 h with gentle shaking. Stem segments (0.6 – 1 cm) of hybrid aspen (4-weeks old culture) were co-cultivated with the mixture of *A. tumefaciens* / acetosyringone for 1-2 h and then transferred to MS medium (pH 5.8). After incubation at 24 ºC for 48 h in dark, segments were washed twice (15 min) with sterile distilled water supplemented with 500 µg $mL^{-1}$ cefotaxime. Segments transformed with either 35S::QsMYB1 or EV were placed on MS medium (pH 5.6) containing 20 g $L^{-1}$ sucrose, 0.1 mg $L^{-1}$ indole-3-butyric acid (IBA), 0.2 mg $L^{-1}$ 6-benzylaminopurine (BAP), 0.01 mg $L^{-1}$ thidiazuron (TDZ), 500 mg $L^{-1}$ cefotaxime and 80 mg $L^{-1}$ kanamycin monosulfate. Besides the EV control, other negative control (or wild type, WT) was performed. The WT control consisted in segments of hybrid aspen subject to the same treatment as both 35S::QsMYB1 and EV control, but without *A. tumefaciens* in the co-cultivation step.The WT segments were cultured in same medium as EV and 35S::QsMYB1 but without kanamycin. A control for kanamycin resistance as the selectable marker was also performed by placing WT segments in medium containing kanamycin (80 mg $L^{-1}$). Cultures were maintained at 22-23 ºC under a 16h/8h photoperiod and sub-cultured every 2 weeks. After shoot initiation (1 - 2 months after transformation), aspen segments were transferred to MS medium (pH 5.6) with 20 g $L^{-1}$ sucrose, 0.1 mg $L^{-1}$ IBA, 0.2 mg $L^{-1}$ BAP, 500 mg $L^{-1}$ cefotaxime and 80 mg $L^{-1}$ kanamycin monosulfate to promote elongation. The concentration of cefotaxime was gradually decreased up to 150 mg $L^{-}1$, before transferring the plants to half-strength MS medium (pH 5.6) to initiate the rooting.

About 2 months after plants being on elongation medium, the T-DNA insertion was confirmed by PCR. DNA of transformed hybrid aspen was isolated from leaves (about 100 mg) using the Nucleospin Plant II kit (Macherey-Nagel) according to manufacturer's instructions. PCR was performed using the primers QsMYB1 forward (5'GGGAGAGCTCCATGTTGTGAC3') and reverse (5' GTTGAGGTACTACCAAGCAAATTAA3') to amplify the *QsMYB1* ORF (1040 bp) and the NPTII forward (5′GAATCGGGAGCGGCGATACCGTAAA3′) and reverse (5′CAAGATGGATTACACGCAGGTTCTC3′) to amplify the neomycin phosphotransferase II (*nptII*) gene originating an amplicon of 700 bp; to eliminate false-positives, the gene *vir*BG was amplified using the primers virBG forward (5′GCGGTGAGACAATAGGCG3′) and reverse (5′GAACTGCTTGCTGTCGGC3′) resulting in a product with 490 bp. PCR cycling conditions were as follow: 95 ºC for 1 min, followed by 35 cycles with 45 s at 95 ºC, 45 s at 58 ºC and 1 min at 72 ºC.

### 2.2. Analysis of transcripts levels

The expression level of *QsMYB1* was evaluated in four selected transgenic lines (L1, L4, L12 and L19) by reverse-transcription quantitative polymerase chain reaction (RT-qPCR). For control, *QsMYB1* expression was also analyzed both in plants transformed with the empty vector (EV) and in non-transformed plants (WT). Total RNA was isolated from one pool of either leaves or stems collected from five different in vitro grown plants (4-weeks old, grown on rooting medium), using the Spectrum Plant Total RNA Kit (Sigma-Aldrich) following manufacturer's instructions. The protocol included also an optional step to remove genomic DNA (gDNA) using the On-column Dnase I digestion set (Sigma-Aldrich). RNA concentration and purity were assessed with a NanoDrop® ND-1000 spectrophotometer (Thermo Scientific) and the integrity was verified on a 1% (w:v) agarose gel. First-strand cDNAs were synthesized from 1.0 µg of total RNA using the QuantiTect Reverse Transcription kit (Qiagen), which includes an additional genomic DNA elimination step and the use of a mix of oligo(dT) and random hexamer primers. A pair of primers was design to specifically amplify QsMYB1, forward (5'AGCCTAAAGCAAGAGATGAAGAGAG3') and reverse (5'ACATCAATATCAGAAAATCTTCCTCCGAG3'), resulting in an amplicon of 190 bp. The PCR mixture included 1X iQ™SYBR® Green Supermix (Bio-Rad Laboratories), 0.2 µM of each primer and 1 µL of the previously synthesized cDNA in a final volume of 20 µL. The RT-qPCR programme consisted in an initial incubation at 95°C for 3 min, followed by 40 cycles with 10 s at 95 ºC, 15 s at 61 ºC and 15 s at 72 °C with a single fluorescent reading taken at the end of each cycle. No template controls (NTC), for each pair of primers, were included in each run. cDNA from each pooled material was used and run in triplicate. To distinguish specific from nonspecific products and primer dimers, a melting curve was obtained immediately after amplification. PCR and melting products were detected in real time with an iCycler iQ5 Instrument (Bio-Rad Laboratories). The threshold cycles ($C_T$) were calculated by the optical interface iQ5 software (Bio-Rad Laboratories. The *Pt1* (POPTR_0002s12910) was used as reference gene and it was amplified with the primers forward (5'GCGGAAAGAAAAACTGCAAG3') and reverse (5'TGACAGCACAGCCCAATAAG3') previously described by Gutierrez et al (2008). PCR conditions and cycling parameters were the same as for *QsMYB1* gene. The same batch of cDNA was used on the amplification of target and reference genes. Primers efficiencies were about 100% calculated by the standard curve method. Relative abundance of *QsMYB1* transcript was calculated by the ΔCt method $2^{(\text{CtReference} - \text{CtTarget})}$ using the *Pt1* gene to normalize samples. Expression levels are represented as fold changes relative to zero.

## 3. Results and discussion

The cDNA sequence of QsMYB1 was cloned for overexpression under control of the 35S CaMV constitutive promoter and transformed in hybrid aspen (Fig 2).

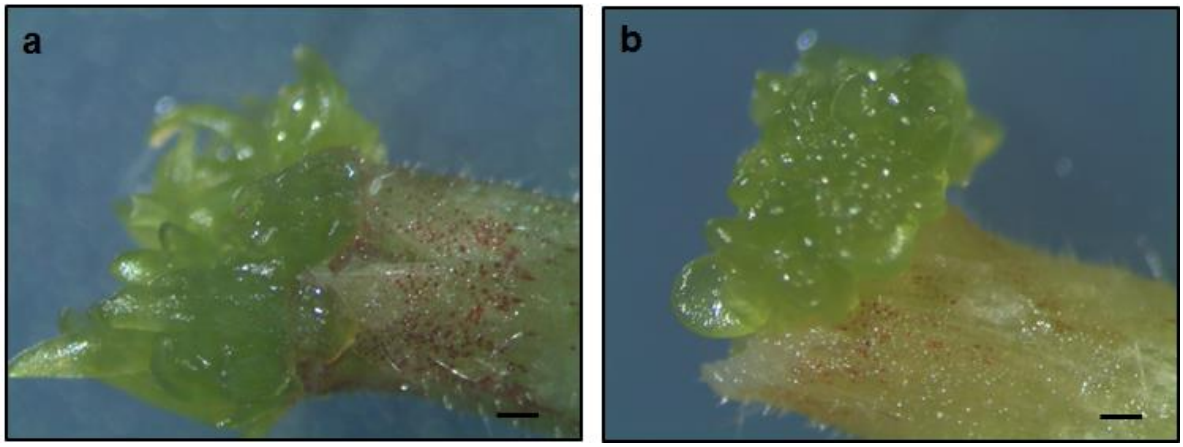


**Fig 2.** Hybrid aspen explants grown in MS medium after shooting initiation. **(a)** WT (1 month after transformation); **(b)** 35::QsMYB1 (2 months after transformation). Bars = 1 mm.

We selected 12 lines (each representing an independent transformation event with 35S::QsMYB1 construct) to test T-DNA insertion by assessing both the amplification of *nptII* gene (selectable marker for kanamycin resistance) and *QsMYB1* ORF (Fig 3). From these 12 lines, only one (L6) seemed to give a negative result regarding insertion of 35::QsMYB1 construct. Four transgenic lines (L1, L4, L12, and L19) were chosen to be evaluated by RT-qPCR in order to determine the relative expression level of *QsMYB1* both in leaves and stems (Fig. 4). Expression analysis showed that among the tested lines, only three of them were effectively overexpressing *QsMYB1*, namely L4, L12 and L19 whereas L1 did not show accumulation of *QsMYB1*. Among the overexpressing lines, L4 and L19 exhibited a much higher accumulation of *QsMYB1* than L12 indicating that the number of transgene copy number may be different between poplar transgenic lines carrying *QsMYB1* gene. Interestingly, all transgenic lines showed a higher *QsMYB1* expression level in leaves than in stem. As it was expected in plants transformed with EV and in WT plants no *QsMYB1* expression was detected.

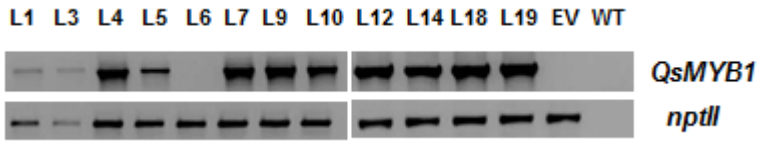


**Fig. 3.** Twelve hybrid aspen lines transformed with 35S::QsMYB1 construct tested for T-DNA insertion by PCR of both kanamycin resistance (*nptII*) and *QsMYB1* genes. Plants transformed with empty vector (EV) and WT plants were also assessed. The amplification of *virBG* gene was also assessed and (as expected) no amplification was detected (data not shown).

Despite confirmation of *QsMYB1* ectopic overexpression, none of three transgenic lines showed visual differences in phenotype when compared to WT neither on elongation (Fig. 5) nor on rooting (Fig. 6) mediums. Plants transformed with the EV also showed a similar phenotype to

WT (Fig 7), indicating that the transformation procedure do not induce any phenotype. A possible explanation for lack of a clear phenotype in QsMYB1 overexpressing lines is that QsMYB1 TF may regulate a pathway that is not induced under normal growth condition or at this developmental stage. Thus, for further functional analysis, transgenic plants should be grown in the greenhouse until they show secondary growth and/or exposed to different stress conditions. The loss function mutant of the QsMYB1 putative orthologous in *Arabidopsis*, the AtMYB68, which is pointed as repressor of the lignin pathway, also did not exhibit a visible phenotype when grown in normal conditions (Feng et al. 2004). But the authors observed that under elevated temperatures the MYB68::GUS reporter was induced, suggesting that MYB68 is modulated by temperature. It is expected that the differences observed in *QsMYB1* transcript levels between the three 35S::QsMYB1 lines, will have implications in the resulting phenotype. Thus, these three overexpressing QsMYB1 transgenic lines represent a valuable resource to perform detailed molecular, physiological and metabolic analyses that will be fundamental to unravel and confirm the QsMYB1 role in cork formation.

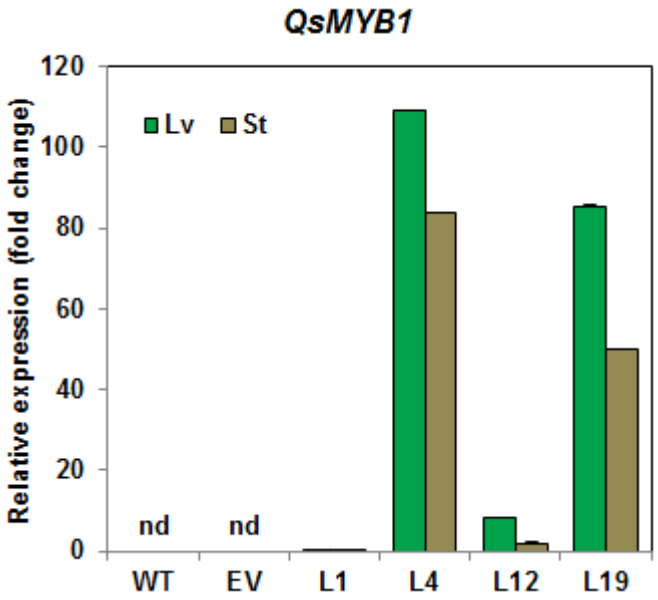


**Fig 4.** Relative expression of *QsMYB1* in leaves (Lv) and stems (St) from transgenic poplar lines harboring the 35::QsMYB1 construct (L1, L4, L12 and L19) evaluated by RT-qPCR. Expression was also analyzed in plants transformed with empty vector (EV) and in WT plants. Expression levels are presented as fold-change relative to zero. Data are means of three technical replicates ± SD. nd, not detected.

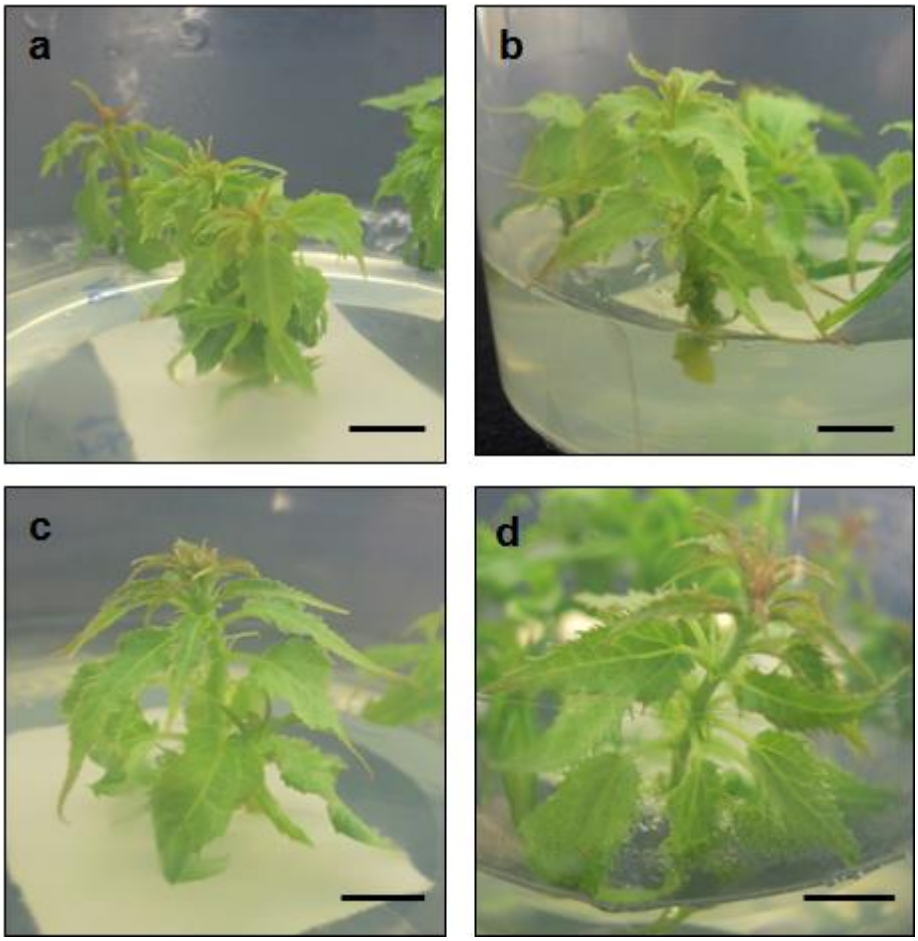


**Fig. 5**. Two-week old in vitro grown (elongation medium) hybrid aspen **(a)** 35::QsMYB1 L12, **(b)** 35::QsMYB1 L19, **(c)** EV, **(d)** WT. Bars = 1 cm


**Fig. 6.** Four-week old in vitro grown (rooting medium) hybrid aspen WT plants and transgenic plants transformed with 35::QsMYB1 (L1, L4, L12 and L19). Bar = 3 cm.

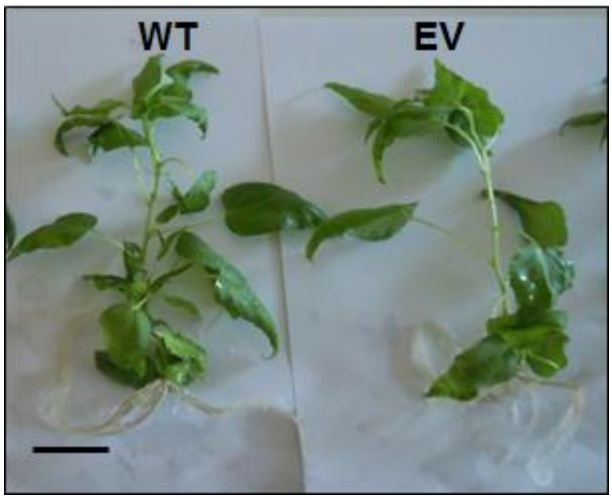


**Fig 7.** Four-week old in vitro grown (rooting medium) hybrid aspen WT and EV plants. Bar = 3 cm.

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# CHAPTER VI

# Concluding remarks and future perspectives

## Conclusions

As continuously highlighted throught this PhD thesis, the molecular mechanisms behind regulation of cork biosynthesis are largely unknown. The tissue availability (restricted to a narrow time frame), the commitment of cork cells in a senescence program and the long reproductive cycle of cork oak are within the main reasons that hamper molecular approaches to cork biosynthesis. However, given its economic importance and the growing need by the industry for raw-cork of high quality, the research on this field has increased, but there is still a long way to go. The first genomic approach to suberin biosynthesis and cork differentiation (Soler et al. 2007), carried out only few years ago, has provided useful data for subsequent investigations. In this PhD thesis, the research was focused on the R2R3-MYB transcription factor identified in that work.

The complete *QsMYB1* gene structure was successfully achieved in Chapter II (Almeida et al. 2013a) and the R2R3-MYB domain was identified. Interestingly, it was also found that a possible alternative splicing mechanism is associated to QsMYB1 regulatory function. Since both splicing variants (*QsMYB1.1* and *QsMYB1.2*) putatively encode a similar protein, the alternative splicing is possibly acting as a posttranscriptional regulatory mechanism that may be modulating either gene expression or translation efficiency. Moreover, the CT dinucleotide repeats within the 5'UTR may be itself a *cis*-regulatory element functioning in regulation of gene expression. Consistent with results from Soler et al. (2007), our results clearly showed that *QsMYB1* variants are up-regulated in cork oak tissues and organs exhibiting secondary growth derived from the activity of cork cambium, mainly in the newly formed cork. The alternative splicing is often tissue and/or development specific. However, in the case of *QsMYB1*, both variants are constitutively expressed in all tissues and organs expressing *QsMYB1*, suggesting that both may be biological meaningful in those tissues. Nevertheless, the biological function of each splicing variant must be explored in the near future. Other functional studies are also needed in order to unravel the cork biosynthesis pathway(s) in which QsMYB1 transcription factor will be acting. One hypothesis that might be considered is that QsMYB1 may have a similar function to its putative orthologous in *Arabidopsis*, the AtMYB68, that was pointed as a putative repressor of lignin (Feng et al. 2004). In fact, some *cis*-regulatory elements related with phenylpropanoid biosynthesis are present in the putative promoter region of *QsMYB1*. Moreover, lignin and suberin, the two main components of cork, share some precursors such as the ones resulting from the phenylpropanoid pathway.

Cork growth is affected by environmental conditions, but the regulation underlying cork oak response to abiotic stresses is still poorly understood. Thus it is of outmost importance to look for regulators acting in this response. In Chapter III, QsMYB1 is presented as a transcription factor possibly involved in this regulatory network (Almeida et al. 2013b). Interestingly, the two stress conditions (cumulative heat and drought) appear to have an opposite effect on *QsMYB1* transcripts. Moreover, results showed that despite the two *QsMYB1* variants being expressed over both stresses, the *QsMYB1.1* (spliced) responds mainly to cumulative heat stress while *QsMYB1.2*

(unspliced) is the most affected by drought. Therefore, it is likely that *QsMYB1* alternative splicing is itself modulated by abiotic stresses, but this should be studied further in the future. The recovery after stresses is not very often evaluated, but here the results showed that recovery of both stresses also induce a differential response by the two spliced variants. Thus, this should be taken in account when similar assays are delineated.

Despite its importance, the hormonal regulation of cork biosynthesis is underexplored and little is known. Chapter IV was dedicated to study the influence of exogenous phytohormones on *QsMYB1* transcripts, analyzed in cork oak stems. The results obtained for MeJa suggest that *QsMYB1* is transiently induced by this phytohormone, mainly the *QsMYB1.2* variant, which opens the possibility that QsMYB1 may be a component of MeJa signaling transduction in cork oak. On the other hand, ABA appears to have no great influence on the expression of *QsMYB1* variants. Concerning IAA, additional work needs to be done to better understand the results. The work described in Chapter IV is the first insight on *QsMYB1* regulation by hormones and only three phytohormones were studied (ABA, MeJa and IAA). But, we consider that this topic should be further explored and the research may be extended to other phytohormones (e.g. JA, ethylene) and it may be worthy to investigate the crosstalk of phytohormones and the response to stresses.

Due to time constraints, it was not possible to complete the reverse genetic approach described in Chapter V. However, the three transgenic poplar lines overexpressing *QsMYB1* will enable a detailed functional characterization that will help to unravel the QsMYB1 function during cork development.

Despite additional work is necessary, the findings presented through this PhD thesis provide relevant data in a subject whose knowledge gap is still significant. Indeed, this is the first study focusing on the characterization of an R2R3-MYB transcription factor up-regulated in cork and potentially involved in the regulatory network of cork oak response to environmental factors and hormone signaling transduction.

## Future perspectives

Some work is already in progress and will be completed in a near future:

- The analysis of the DNA methylation of cytosines in promoter, 5'UTR and coding region from the samples of drought and heat stresses assays. This is being done by treating the genomic DNA (from stem) with sodium bissulfite and sequencing.

- The analysis of the 5'UTR-SSR polymorphism in a population of cork oak trees (about 70).

- The metabolic and proteomic profiles of samples (leaves) from both abiotic stresses assays are being performed.

- A trans-activation assay will be carried out in yeast to investigate wether QsMYB1 acts as an activator and/or repressor. A plasmid expressing QsMYB1 fused to LexA DNA-binding domain was already accomplished and will be used together with a *lacZ* reporter plasmid.

Besides this ongoing work, the findings of this PhD thesis also open the possibility to future research that will be fundamental to answer some other questions that remained unanswered. The future research should focus mainly on QsMYB1 functional analysis and several possible research directions are mentioned below.

- Proceed with the analysis of the transgenic poplar lines obtained in Chapter V would be a good start point to gain new insights into pathway(s) in which QsMYB1 may be involved. These analyses can include: the comparison of transgenic with wild-type poplar plants at chemical (e.g. suberin and lignin content) and ultra-structural (analysis of periderm) levels; comparative metabolomics study between transgenic and wild-type plants; comparison of expression pattern of some lignin and suberin related genes in transgenic and wild-type plants.

- The biological meaning of the two alternative splicing variants also needs to be clarified. This may be accomplished by performing heterologous transformation with constructs harboring the *QsMYB1* promoter and the 5'UTR (with or without the intron) fused with a reporter protein.

- Given the results obtained in Chapter III and IV, it will be also important to perform functional analysis in order to assess if the promoter of *QsMYB1* is responsive to abiotic stresses and/or hormones. For this, transgenic plants harboring the *QsMYB1* promoter fused to a reporter protein should be used in abiotic stress or hormone assays.

- In a more distant future, it would be of outmost importance to identify the putative target genes of QsMYB1 transcription factor to better understand the regulatory network integrated by QsMYB1.

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